JOURNAL OF AGRICULTURAL RESEARCH

VOL. 54 WASHINGTON, D. C., APRIL 15, 1937 No. 8

ALFALFA LEAF MEAL AS A SOURCE OF VITAMIN A FOR GROWING CHICKENS[1]

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INTRODUCTION

In the fall of 1929 a study of alfalfa products as sources of vitamin A for growing chickens was begun at the Southwest Poultry Experiment Station of the United States Department of Agriculture, Glendale, Ariz. Simple basal diets, deficient in protein as well as in vitamin A, were used in the first few experiments, the results of which have been presented elsewhere.[2] When these basal diets were fed without vitamin A supplements, the chicks grew very slowly, but in some cases a few were able to survive as long as 20 weeks. When various vitamin A supplements were added, the gains in live weight were dependent to a great extent on the protein content of the supplements. In some instances, when enough vitamin A was supplied, nearly all the chicks remained alive for 20 weeks or longer. Although gain in live weight could not be used as a measure of vitamin A potency, it was possible to make rough comparisons of the vitamin A potency of the supplements by comparing the average number of days the chicks survived.

The results of these first experiments clearly indicated the desirability of devising a more suitable basal diet—one which would contain sufficient protein of good quality so that the protein supplied by the vitamin A supplements would have little or no effect on growth. Such a diet was devised, and this paper presents data obtained in a study of this diet, as well as data on the relative value of different alfalfa leaf meals as sources of vitamin A for growing chickens.

EXPERIMENTAL PROCEDURE

In the study reported in this paper seven experiments were conducted. The first was begun in October 1931, and the last in June 1934. The lengths of these experiments varied from 8 to 20 weeks, and in one case (experiment 5) the birds were continued under observation until they were 30 weeks old.

At the beginning of each experiment the chicks were distributed at random among the pens. In any given experiment the same number of chicks were placed in each pen, but the number per pen varied from experiment to experiment and depended on the number of apparently strong, healthy chicks which were available at the time. The smallest number of chicks per pen in any of the experiments was

[1] Received for publication Dec. 16, 1936; issued May, 1937.

[2] HEYWANG, B. W., and TITUS, H. W. SOURCES OF VITAMIN A, PARTICULARLY ALFALFA PRODUCTS, FOR MAINTAINING THE LIFE OF CHICKS. Poultry Sci. 11 (4): 234-238, illus. 1932.

35 and the largest number was 50. Single-Comb White Leghorn chicks were used in the first and seventh experiments, and Single-Comb Rhode Island Red chicks in the other five experiments.

All chicks were weighed when they were 1 day old and at age intervals of 2 weeks thereafter until they died or until the experiments were ended. A record was kept of the feed consumed during each 2 weeks by the chicks in all the pens.

The pens were of a size suitable for holding 50 chicks. In experiments 1 and 3 they were located in a long brooder house and in the other experiments in separate brooder houses which were of equal size and similar in every way. Electrically heated brooders were used.

In the first experiment an attempt was made to obtain better growth by modifying one of the original basal diets (hereinafter referred to as the old basal diet) by substituting 5 percent of each of dried buttermilk and beef scrap for equal quantities of white corn meal. Since improvement in growth was not so great as was desired, for the second experiment another basal diet (hereinafter referred to as the new basal diet) was devised. For the purpose of the experiment, this basal diet seemed to be satisfactory, but there remained the possibility that it was slightly deficient in vitamin G. Accordingly, in the third experiment graded quantities of pure dried yeast and of dried whey were added to the new basal diet to ascertain whether it contained an adequate supply of this vitamin. The results of this experiment eliminated the possibility that the new basal diet was deficient in vitamin G.

The fourth experiment was conducted for the purpose of comparing the effect of different graded quantities of alfalfa leaf meal when added to both the new and the old basal diets. In the fifth, sixth, and seventh experiments graded quantities of alfalfa leaf meals from different sources were added to the new basal diet for the purpose of comparing their relative value as sources of vitamin A.

In experiment 2 a pen was included which received a 10-minute daily exposure to direct sunshine. This was done for the purpose of determining whether the birds receiving sunlight as a source of vitamin D would live longer than those receiving viosterol solution. Since no difference was observed both pens may be considered negative controls.

The two basal diets were compounded as shown in table 1.

TABLE 1.—*Basal diets used in the experiments*

Item	Old basal diet	New basal diet
	Percent	*Percent*
White corn meal	90.0	64.0
Wheat middlings		15.0
Casein		10.0
Pure dried yeast		4.0
A-P yeast foam	6.0	
Irradiated yeast (70 D)	.5	
Ground limestone	2.0	3.0
Steamed bonemeal	1.0	1.0
Viosterol solution (50 D)		2.0
Common salt	.5	1.0
Total	100.0	100.0

When cod-liver oil was used as a source of vitamin A in the positive control pens, it was substituted for an equal weight of viosterol solution.

In most of the experiments both positive and negative control pens were used, that is, chicks in one or two pens received the basal diet supplemented with cod-liver oil and those in one or two others received the basal diet without any vitamin A supplement. For further comparative purposes, in each of the first two experiments there was included a pen of chicks which received some freshly cut alfalfa and in the second experiment a pen of chicks which received fresh carrots, as a source of vitamin A. In the third experiment all the chicks received 2 percent of cod-liver oil. All other chicks in the seven experiments received small quantities of alfalfa leaf meal. The individual diets were compounded by mixing an appropriate number of parts of the vitamin A supplement with enough of the basal diet to make 100 parts, rather than by adding a given number of parts of the supplement to each 100 parts of the basal diet.

Alfalfa leaf meals produced in different sections of the country and cured, or dried, by several different methods were studied. Most of these meals were bought on the open market, but several of them were obtained directly from the manufacturer. As much information as possible was obtained about each of the samples. This information, together with similar information for the samples of fresh alfalfa, follows.

ARIZONA SUN-CURED ALFALFA LEAF MEAL.

Sample 1: Used in experiment 1; a product of local manufacture; fed at levels of 5 and 10 percent of the diet.

Sample 2: Used in experiments 2 and 5; a product of local manufacture; used a few months after it was prepared; fed at a level of 7.5 percent of the diet in experiment 2 and at levels of 2.5 and 5 percent in experiment 5.

Sample 3: Used in experiment 4; a product of the same source as sample 2; contained approximately 22 percent of protein and 16 percent of crude fiber; fed at levels of 1.5, 3, and 6 percent with the new basal diet and at levels of 1.5, 3, 6, and 12 percent with the old basal diet.

CALIFORNIA SUN-CURED ALFALFA LEAF MEAL.

Sample 4: Used in experiments 2 and 5; obtained from a manufacturer in Van Nuys, Calif.; made from the fifth cutting of the Chilean variety, harvested in September; used in experiment 2 about 5 months after it was prepared and in experiment 5 about 7 months after preparation; fed at a level of 7.5 percent of the diet in experiment 2, and at levels of 2.5 and 5 percent in experiment 5.

Sample 5: Used in experiment 6; obtained from the same manufacturer as sample 4; made from fourth cutting of the Chilean variety; contained about 21 percent of protein and was graded as U. S. No. 1 Extra Fine alfalfa leaf meal; fed at levels of 0.5, 1.5, 2.5, and 7.5 percent of the diet.

Sample 6: Used in experiment 7 about 9 months after it was prepared; contained about 21 percent of protein; fed at levels of 2.5 and 5 percent of the diet.

Sample 7: Used in experiment 7 about 9 months after it was prepared; contained about 17.3 percent of protein; fed at levels of 2.5 and 5 percent of the diet.

COLORADO SUN-CURED ALFALFA LEAF MEAL

Sample 8: Used in experiments 2 and 5; obtained from a manufacturer in Lamar, Colo.; made from a mixture of one part of first-cutting and two parts of third-cutting alfalfa hay which had been in the stack about 3 months before it was milled; fed at a level of 7.5 percent of the diet in experiment 2 and at levels of 2.5 and 5 percent in experiment 5.

CALIFORNIA "ALFALFA POWDER"

Sample 9: Used in experiments 2 and 5; obtained from a manufacturer in Burbank, Calif.; made from fourth cutting of alfalfa harvested in the bud in November; run through a dehydrator ("top-of-flame gas") about 5 hours after it was cut; fed at a level of 7.5 percent of the diet in experiment 2 and at levels of 2.5 and 5 percent in experiment 5.

CALIFORNIA DEHYDRATED ALFALFA LEAF MEAL

Sample 10: Used in experiment 1; prepared by a manufacturer in Van Nuys, Calif.; fed at levels of 2.5 and 5 percent of the diet.

Sample 11: Used in experiments 2 and 5; prepared by the same manufacturer as sample 10; made from fifth cutting of the Chilean variety, harvested in September; fed at a level of 7.5 percent of the diet in experiment 2 and at levels of 2.5 and 5 percent in experiment 5.

Sample 12: Used in experiment 6; obtained from the same manufacturer as sample 2; made from the same alfalfa (same cutting from the same field) as that used in sample 2 (a sun-cured meal); contained about 21.6 percent of protein and was graded as U. S. Extra Green Extra Fine alfalfa leaf meal; fed at levels of 0.5, 1.5, 2.5, 5, and 7.5 percent of the diet.

Sample 13: Used in experiment 7 about 3 months after it was prepared; contained about 14.1 percent of protein; fed at levels of 2.5 and 5 percent of the diet.

Sample 14: Used in experiment 7 about 3 months after it was prepared; contained about 19.4 percent of protein; fed at levels of 2.5 and 5 percent of the diet.

OKLAHOMA DEHYDRATED ALFALFA LEAF MEAL

Sample 15: Used in experiment 1; obtained from a manufacturer in Paul's Valley, Okla.; fed at levels of 5 and 10 percent of the diet.

FRESH ALFALFA

Sample 16: Used in experiment 1; raised locally; fed at a level of 4.75 percent (dry basis) immediately after cutting.

Sample 17: Used in experiment 2; raised locally; fed at a level of 7.5 percent (dry basis) immediately after cutting.

EXPERIMENTAL DATA AND DISCUSSION

As was previously pointed out, satisfactory growth was not obtained in earlier experiments with the old basal diet.[3] Therefore, in experiment 1 of the present series an attempt was made to improve it by including small quantities of dried buttermilk and beef scrap. Since the results were not fully satisfactory, the so-called new basal diet was

[3] HEYWANG, B. W., and TITUS, H. W. See footnote 2.

devised. The results of experiment 1 which deal with the main purpose of the study are presented in table 5, with other data for comparison.

In order to show the ability of the new basal diet to support growth when it was adequately supplemented with various sources of vitamin A, the average live weights, at 8 and 16 weeks of age, of the chicks in the several pens of experiment 2 are presented in table 2. In this table, and likewise in tables 3 and 4, the errors shown are the standard errors. The probable error is 0.6745 times the standard

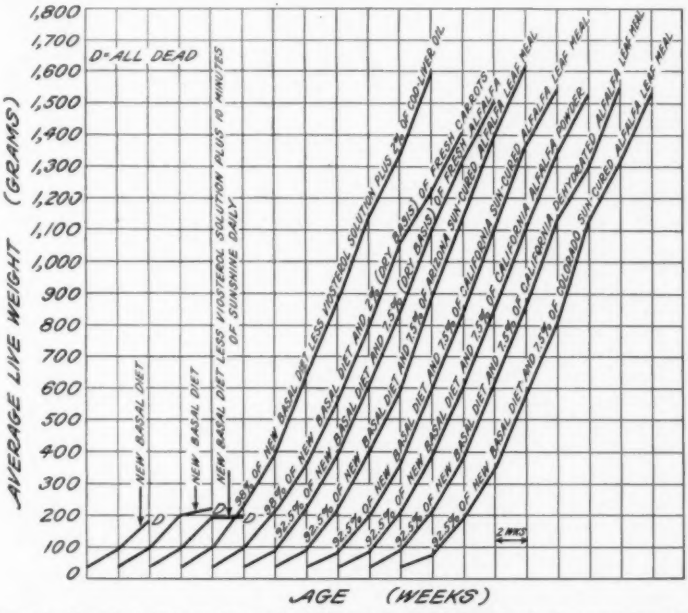
AVERAGE LIVE WEIGHT (GRAMS)
1,800
1,700
1,600
1,500
1,400
1,300
1,200
1,100
1,000
900
800
700
600
500
400
300
200
100
0
D-ALL DEAD
NEW BASAL DIET
NEW BASAL DIET
NEW BASAL DIET LESS VIOSTEROL SOLUTION PLUS 10 MINUTES OF SUNSHINE DAILY
50% OF NEW BASAL DIET LESS VIOSTEROL SOLUTION PLUS 2% OF COD-LIVER OIL
90.5% OF NEW BASAL DIET AND 2% (DRY BASIS) OF FRESH CARROTS
92.5% OF NEW BASAL DIET AND 7.5% (DRY BASIS) OF FRESH ALFALFA
92.5% OF NEW BASAL DIET AND 7.5% OF ARIZONA SUN-CURED ALFALFA LEAF MEAL
92.5% OF NEW BASAL DIET AND 7.5% OF CALIFORNIA SUN-CURED ALFALFA LEAF MEAL
92.5% OF NEW BASAL DIET AND 7.5% OF CALIFORNIA ALFALFA POWDER
92.5% OF NEW BASAL DIET AND 7.5% OF CALIFORNIA DEHYDRATED ALFALFA LEAF MEAL
92.5% OF NEW BASAL DIET AND 7.5% OF COLORADO SUN-CURED ALFALFA LEAF MEAL
2 WKS.
AGE (WEEKS)

FIGURE 1.—Growth of chicks on the new basal diet when unsupplemented and when supplemented with various sources of vitamin A (experiment 2).

error. Growth curves of these pens of chicks are given in figure 1. In those pens in which the chicks received no vitamin A supplement none of the chicks survived to the end of the eighth week, but chicks receiving the supplement made good growth.

For experiment 3, in which the effect, on growth, of including graded quantities of vitamin G supplements was studied, all the pertinent data are presented in table 3. The growth curves of the chicks are shown in figure 2.

In experiment 4, in which the two basal diets, both unsupplemented and supplemented with sources of vitamin A, were compared, the average live weights at 8 and 20 weeks are given (table 4). The growth curves are shown in figure 3. When the unsupplemented new basal diet was fed, none of the chicks survived to the end of the eighth week, but when the unsupplemented old basal diet was fed, a

few chicks survived as long as 12 weeks. The growth curves in figure 3 for the chicks on the unsupplemented old basal diet is ex-

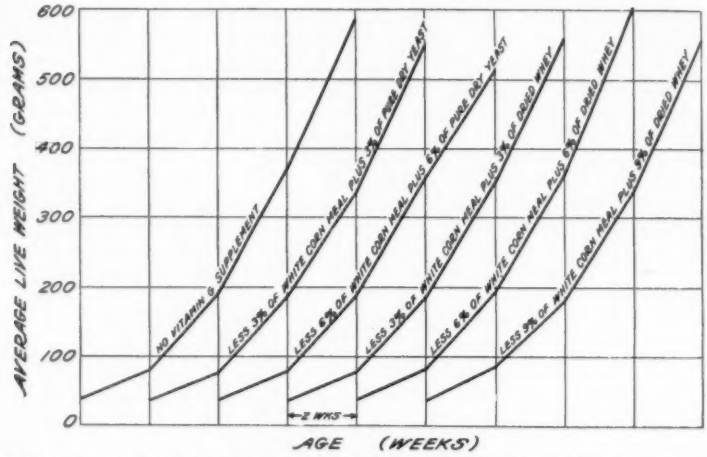
AVERAGE LIVE WEIGHT (GRAMS)
600
500
400
300
200
100
0
NO VITAMIN G SUPPLEMENT
LESS 3% OF WHITE CORN HEAL PLUS 3% OF PURE DRY YEAST
LESS 6% OF WHITE CORN HEAL PLUS 3% OF PURE DRY YEAST
LESS 3% OF WHITE CORN HEAL PLUS 3% OF DRIED WHEY
LESS 6% OF WHITE CORN HEAL PLUS 3% OF DRIED WHEY
LESS 3% OF WHITE CORN HEAL PLUS 3% OF DRIED WHEY
2 WKS
AGE (WEEKS)

FIGURE 2.—Growth of chicks on the new basal diet and when supplemented with graded quantities of sources of vitamin G (experiment 3).

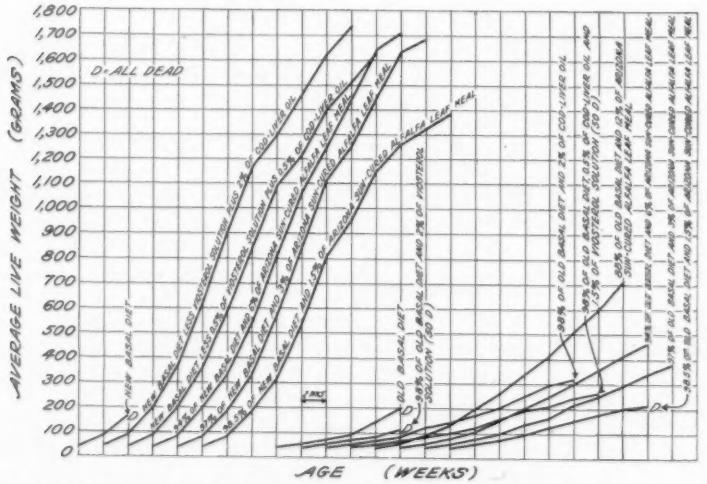

FIGURE 3.—Growth of chicks on the old and new basal diets when unsupplemented and when supplemented with sources of vitamin A (experiment 4).

tended to only the tenth week because only a small number survived to the end of the twelfth week.

TABLE 2.—*Influence of various vitamin A supplements on the average live weights of Rhode Island Red chicks at 8 and 16 weeks of age (experiment 2)*

Supplement	Sample no.	Quantity of supplement included in diet	Average live weight of chicks at 8 weeks [1]	Average live weight of chicks at 16 weeks [1]
		Percent	*Grams*	*Grams*
None (new basal diet only)			[2]	
Do			[2]	
None (new basal diet less viosterol solution plus 10 minutes of sunshine daily)			[2]	
Cod-liver oil		2.0	632.8±14.5	1,599.7±28.7
Fresh carrots		[3] 2.0	560.7±15.8	1,401.9±31.5
Fresh alfalfa	17	[3] 7.5	609.0±16.3	1,506.0±32.0
Arizona sun-cured alfalfa leaf meal	2	7.5	590.6±15.7	1,616.8±30.7
California sun-cured alfalfa leaf meal	4	7.5	597.4±14.5	1,545.5±28.4
California alfalfa powder	9	7.5	596.1±15.1	1,530.7±30.5
California dehydrated alfalfa leaf meal	11	7.5	612.4±14.8	1,550.4±29.2
Colorado sun-cured alfalfa leaf meal	8	7.5	571.5±15.0	1,530.6±29.7

[1] Unweighted average of weights of males and females.
[2] No chicks survived to end of eighth week.
[3] Dry basis.

TABLE 3.—*Influence of vitamin G supplements on mortality, feed consumption, cumulative efficiency of the feed, and average live weights of Rhode Island Red chicks at 8 weeks of age (experiment 3)*

Supplement [1]	Quantity of supplement added to diet [2]	Mortality of chicks	Feed consumed per chick	Gain in live weight per gram of feed consumed	Average live weight at 8 weeks [3]
	Percent	*Percent*	*Grams*	*Grams*	*Grams*
None (new basal diet only)		9.09	1,485	0.370	586.6±16.8
Pure dried yeast	3	4.55	1,465	.353	549.6±15.6
	6	11.36	1,505	.319	514.2±17.2
Dried whey	3	18.18	1,557	.339	556.8±17.0
	6	29.55	1,625	.346	602.3±15.8
	9	2.27	1,520	.349	559.1±15.7

[1] In order to be sure that an adequate quantity of vitamin A would be supplied, 2 percent of cod-liver oil was substituted for the 2 percent of viosterol solution 50 D in all the diets.
[2] The vitamin G supplements were substituted for an equal weight of white corn meal in the basal diet.
[3] Unweighted average of weights of males and females.

TABLE 4.—*Influence of various vitamin A supplements on the efficiency of the new and old basal diets, as indicated by the average live weights of Rhode Island Red chicks at 8 and 20 weeks of age (experiment 4)*

Basal diet and supplement	Sample no.	Quantity of supplement included in diet	Average live weight at 8 weeks [1]	Average live weight at 20 weeks [1]
		Percent	*Grams*	*Grams*
New:				
None (basal diet only)			[2]	
Cod-liver oil		2.0	618.7±13.0	1,733.4±38.4
Cod-liver oil plus 1.5 percent of viosterol solution 50 D		.5	591.1±12.2	1,629.7±36.8
Arizona sun-cured alfalfa leaf meal	3	6.0	543.6±12.2	1,706.4±35.9
	3	3.0	573.0±12.2	1,686.1±35.9
	3	1.5	537.2±12.7	1,384.6±50.0
Old:				
None (basal diet only)			143.3± 6.9	[2]
None (basal diet plus 2 percent of viosterol solution 50 D)			110.7± 8.0	[2]
Cod-liver oil		2.0	121.5± 3.4	318.0±15.2
Cod-liver oil plus 1.5 percent of viosterol solution 50 D		.5	99.1± 3.5	261.4±17.9
Arizona sun-cured alfalfa leaf meal	3	12.0	196.7± 3.4	713.5±14.5
	3	6.0	150.0± 3.7	466.2±15.3
	3	3.0	136.2± 3.4	377.7±18.5
	3	1.5	124.7± 3.6	[2]

[1] Unweighted average of weights of males and females. [2] No chicks survived to the end of this period.

The effect, on viability and growth, of the inclusion in the diet of the various vitamin A supplements as observed in all the experiments except experiment 3, is shown in table 5. For both viability and growth the following ratings are given: Excellent, good, fair, poor, and very poor. These ratings, although having their usual meanings, were so made that the difference between any two consecutive ones is somewhat less than is considered statistically significant (odds of 19 to 1), but the difference between any one and the second consecutive one is significant (odds of at least 19 to 1), and the difference between any one and the third consecutive one is highly significant (odds of at least 99 to 1). It should be borne in mind, however, that in a few cases the difference between any rating and the second consecutive one may lack but little of being highly significant.

The data presented in the tables show that the new basal diet was capable of supporting good growth, when it was adequately supplemented with a source of vitamin A. They further indicate that the additional protein supplied by the alfalfa products had no observable effect on growth. On the other hand, when the old basal diet was used, the protein supplied by the alfalfa products had a pronounced effect on growth. This is strikingly shown in figure 3.

According to the data presented in table 2, no marked beneficial effect on growth, mortality, or utilization of the feed was observed as a result of the addition, to the new basal diet, of graded quantities of vitamin G supplements. As a matter of fact, increasing the quantity of yeast in the diet seemed to decrease the rate of growth. Increasing the quantity of dried whey, as a source of vitamin G, had no consistent effect on growth.

The fact that the new basal diet contained an adequate supply of protein of good quality and of vitamin G is of considerable importance when a vitamin A-deficient diet is to be used in studying the vitamin A potency of alfalfa products, because alfalfa, besides containing vitamin A, also contains protein of excellent quality and is a good source of vitamin G.

It is noteworthy that the chicks were able to live appreciably longer on the unsupplemented old basal diet than on the unsupplemented new basal diet. They grew very slowly on the former diet, whereas on the latter they grew rather rapidly. This observation suggests that the process of growth uses up a rather large part of the total quantity of vitamin A required by a growing chick. Such an inference is in agreement with the practice in making vitamin A assays of using gain in live weight as a measure of vitamin A potency. However, it must be concluded that a part of the vitamin A requirement of a growing animal is used for maintenance. This conclusion and the knowledge that there is a limit to the growth that an animal can make during a given period account for the fact that the relationship between vitamin A intake and growth is not expressible by a linear equation.

In experiment 2 (table 5) it was found that 2 percent, on a dry basis, of carrots did not supply enough vitamin A for good viability and that growth to the seventeenth week was only fair. Even 7.5 percent, on a dry basis, of fresh alfalfa failed to permit the chicks to grow as well as did 2 percent of cod-liver oil.

TABLE 5.—*Effect of vitamin A supplements on the viability and growth of chicks*[1] *(experiments 1–7)*

Supplement	Sample no.	Quantity of supplement included in diet	Experiment no.	Viability to 8 weeks of age	Growth to 8 weeks of age	Viability to 20 weeks of age[2]	Growth to 20 weeks of age[2]
		Percent					
Arizona sun-cured alfalfa leaf meal	1	5.0	1	VP	F	VP	P
	1	10.0	1	P	F	P	E
	2	2.5	5	P	E	VP	VP
	2	5.0	5	E	E	F	VP
	2	7.5	2	E	E	E	E
	3	1.5	4	G	F	VP	P
	3	3.0	4	E	G	E	E
	3	6.0	4	E	F	E	E
California sun-cured alfalfa leaf meal	4	2.5	5	E	E	E	G
	4	5.0	5	E	E	G	E
	4	7.5	2	E	E	E	G
	5	.5	6	VP	P	AD	AD
	5	1.5	6	VP	P	VP	P
	5	2.5	6	G	G	P	G
	5	5.0	6	G	F	F	F
	5	7.5	6	E	E	F	E
	6	2.5	7	F	E	F	G
	6	5.0	7	E	E	(3)	(3)
	7	2.5	7	F	E	G	VP
	7	5.0	7	G	G	G	G
Colorado sun-cured alfalfa leaf meal	8	2.5	5	E	G	E	E
	8	5.0	5	E	F	G	E
	8	7.5	2	G	G	G	G
California alfalfa powder	9	2.5	5	VP	P	VP	P
	9	5.0	5	E	E	E	E
	9	7.5	2	G	E	G	G
California dehydrated alfalfa leaf meal	10	2.5	1	E	G	G	G
	10	5.0	1	E	E	E	E
	11	2.5	5	E	E	G	E
	11	5.0	5	E	E	G	G
	11	7.5	2	E	E	G	G
	12	.5	6	VP	P	VP	P
	12	1.5	6	E	E	F	G
	12	2.5	6	E	E	F	F
	12	5.0	6	E	E	F	E
	12	7.5	6	E	E	G	F
Oklahoma dehydrated alfalfa leaf meal	13	2.5	7	P	E	AD	AD
	13	5.0	7	G	E	F	E
	14	2.5	7	E	F	E	E
	14	5.0	7	E	F	G	E
	15	5.0	1	E	G	E	E
	15	10.0	1	G	G	F	E
Fresh alfalfa	16	[4] 4.75	1	VP	F	VP	P
	17	[4] 7.5	2	E	E	E	G
Cod-liver oil	----	.5	4	E	E	G	G
	----	2.0	4	F	E	F	E
	----	2.0	2	E	E	G	F
	----	2.0	2	G	E	(3)	(3)
	----	2.0	5	E	F	E	G
	----	2.0	6	E	E	G	E
	----	2.0	7	F	G	G	F
Carrots (fresh)	----	[4] 2.0	2	P	G	P	F
No supplement (new basal diet only)	----	0	2	AD	AD	AD	AD
	----	0	2	AD	AD	AD	AD
	----	0	4	AD	AD	AD	AD
	----	0	5	VP	VP	AD	AD
	----	0	6	AD	AD	AD	AD
	----	0	7	AD	AD	AD	AD
No supplement (new basal diet and 10 minutes of sunshine daily)	----	0	2	AD	AD	AD	AD

[1] E=excellent, G=good, F=fair, P=poor, VP=very poor, and AD=all dead.
[2] Experiment 2 was conducted for only 16 weeks.
[3] Discontinued at the end of 14 weeks.
[4] Dry basis.
[5] Discontinued at the end of 12 weeks.

In experiment 4 viability on the new basal diet was just as good when 0.5 percent of cod-liver oil was used as when 2 percent was used but growth to the twenty-first week was somewhat inferior. On the old basal diet the relative difference in growth, when these two levels of cod-liver oil were used, was much greater.

The data given in table 5 clearly show that the vitamin A potency of alfalfa leaf meals varies considerably. It is apparent that there is about as much variability within a given type of alfalfa leaf meal as there is among the different types studied.

In the case of Arizona sun-cured alfalfa leaf meals one sample supplied an adequate quantity of vitamin A when fed at a level of 3 percent, but another sample failed to do so at a level of 5 percent. In some instances 2.5 percent of California sun-cured alfalfa leaf meal supplied enough vitamin A, but in at least one case this quantity was insufficient. In the one instance in which Colorado sun-cured alfalfa leaf meal was fed at a level of 2.5 percent of the diet, excellent results were obtained.

In general, the data indicate that the dehydrated meals are better than the sun-cured; nevertheless, 2.5 percent of one sample of California dehydrated alfalfa leaf meal failed to supply enough vitamin A to permit any of the chicks to remain alive for a period of 20 weeks.

From the data presented it may be concluded that although as little as 1.5 percent of alfalfa leaf meal may supply an adequate quantity of vitamin A for good viability and growth it would be unwise to depend on less than 5 percent of any given meal unless it previously had been shown to supply enough vitamin A at some lower level.

Guilbert and Hart,[4] as a result of a study of the vitamin A requirement of cattle, recently advanced the hypothesis that "vitamin A requirement is related to body weight rather than energy requirement" and state that "this implies that to compensate for lower food consumption per unit of weight, large animals require a higher percentage of vitamin A in the diet than do small animals." This implication is verified by some of the results presented in table 5, inspection of which shows that there were several cases in which the supplement apparently supplied sufficient vitamin A to the ninth week for good viability and growth but failed to supply enough to the seventeenth or twenty-first week.

In experiment 5, in which the chicks were kept under observation until the survivors were 30 weeks old, it was observed that although about 85 percent of the chicks receiving 5 percent of an Arizona sun-cured alfalfa leaf meal were alive at 20 weeks, none survived for 30 weeks. In the same experiment about 56 percent of the chicks receiving 2.5 percent of the California alfalfa powder survived 20 weeks but only about 26 percent survived 30 weeks. In another case about 80 percent of the chicks receiving 2.5 percent of an Arizona sun-cured alfalfa leaf meal survived 12 weeks but none were alive at the end of the twenty-second week.

SUMMARY AND CONCLUSIONS

A series of seven experiments was conducted with growing chicks for the purpose (1) of devising a suitable vitamin A deficient diet for studying the vitamin A potency of alfalfa products and (2) of comparing the vitamin A potency of samples of alfalfa leaf meals produced in different sections of the country and cured, or dried, by different methods. The basal diet used in comparing the vitamin A potency of the alfalfa products contained sufficient protein of good

[4] GUILBERT, H. R., and HART, G. H. MINIMUM VITAMIN A REQUIREMENTS WITH PARTICULAR REFERENCE TO CATTLE. Jour. Nutrition 10: 409–427. 1935.

quality so that the protein supplied by the vitamin A supplements had no observable effect on growth. This diet also contained an adequate quantity of vitamin G.

It is concluded that the vitamin A potency of alfalfa leaf meals varies greatly and that there is about as much variability within a given type of alfalfa leaf meal as there is among the different types studied. Although as little as 1.5 percent of alfalfa leaf meal may supply an adequate quantity of vitamin A for good viability and growth, it is unwise to depend on less than 5 percent of an alfalfa leaf meal of unknown potency.

Evidence was obtained in support of Guilbert and Hart's suggestion that large animals require a higher percentage of vitamin A in the diet than do small animals.

COMPARISON OF COD-LIVER OIL AND ULTRAVIOLET IRRADIATION AS SOURCES OF VITAMIN D FOR CONFINED LAYING HENS[1]

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INTRODUCTION

In recent years various problems in poultry management have caused the poultry industry to resort more and more to semiconfinement and close confinement for the rearing and maintaining of its poultry flocks. This change of procedure from the raising of chickens on free range has brought new problems to the poultryman and, consequently, to the research worker who is endeavoring to help the poultryman out of his difficulties.

Vitamin D, a nutrient supplied to animals through the action of sunshine, is essential to the proper development of growing chickens and the normal processes of mature birds. Since birds in strict confinement are deprived of the ultraviolet rays from sunshine, they must obtain vitamin D from some other source. The object of the study reported in this paper was to determine the relative value of cod-liver oil and ultraviolet irradiation for supplying vitamin D to laying hens held in strict confinement, when the composition of the diet is kept constant throughout the year.

In 1931 Hendricks, Lee, and Godfrey (*4*)[2] reviewed previous investigations on the use of cod-liver oil and ultraviolet irradiation for supplying vitamin D to confined birds and reported an experiment similar to the one now described. In their work, however, the mineral supplement was modified several times during each year, and changes were made in the source of irradiation. They concluded (*4*, p. 533):

> Feeding cod-liver oil or administering ultraviolet irradiation to laying hens confined without access to direct sunlight or green feed increased egg production and the thickness of the egg shells and improved the general condition of the birds.

They pointed out that both of these sources of vitamin D seemed to have a beneficial effect on egg weight and hatchability.

Later investigations, such as that reported by Branion and Smith (*1*) in 1932, confirmed the importance of vitamin D as a factor in egg production and hatchability. Edson (*3*), after a study of 3 years, concluded that hatchability is improved considerably when cod-liver oil is fed to confined pullets. Reid (*6*) in 1933 discussed the possibilities of using ultraviolet irradiation as a source of vitamin D for laying hens. Murphy, Hunter, and Knandel (*5*) used fortified cod-liver oil as the source of vitamin D and reported in 1934 that when an adequate quantity of vitamin D is supplied, egg size and the weight of eggshells are greater than when the supply of vitamin D is inadequate. In the same year Carver and his associates (*2*) showed that the lack of sufficient quantities of vitamin D from either cod-liver oil or sun-

[1] Received for publication Dec. 16, 1936; issued May 1937.

[2] Reference is made by number (italic) to Literature Cited, p. 582.

shine seriously retards normal egg production, egg weight, and good hatchability. Titus and Nestler (*9*) in 1935 studied the effect on laying hens of cod-liver oil and viosterol at various dietary levels, and concluded that the optimum level of cod-liver oil in the diet of strictly confined laying stock is between 1 and 2 percent.

EXPERIMENTAL METHODS AND MATERIALS

The experiment reported in this paper was carried on for nearly 2 years at the National Agricultural Research Center, Beltsville, Md. The six pens used in this study were located on the second floor of a frame building, and each was approximately 8 by 11 feet in size. The only sunlight reaching them was filtered through ordinary window glass.

On September 6, 1929, 90 Rhode Island Red pullets were selected from those on the growing range and distributed at random, 15 to a pen. One cockerel was placed in each of the pens, and twice a week these cockerels were transferred from one pen to the next. All the females alive and in good condition at the end of the first year were retained for the second year's work.

On September 6, 1930, the number of birds per pen was brought to 15 again by the addition of Rhode Island Red yearlings that had been fed the same diet in another experiment. These birds were added in order to maintain the same area of floor space and hopper space per bird in each pen. No data for these birds, except their feed consumption, are included in the paper.

Two all-mash diets differing only in mineral content were fed throughout the experiment. Diet 1 was used in all pens from the beginning of the experiment to September 6, 1930, a period of 52 weeks, and diet 2 was fed during the remaining 48 weeks of the experiment. The change in the mineral content was made on the supposition that yearling hens require less phosphorus and more calcium in their feed than pullets. The essential differences between the diets were that diet 2 contained one and one-half times as much ash as diet 1 and that the former diet had 0.8 percent of phosphorus and 3.2 percent of calcium, whereas the latter had 1.0 percent of phosphorus and 2.1 percent of calcium. These diets were compounded as shown in table 1. The proximate chemical analyses of these diets was as shown in table 2.

TABLE 1.—*Composition of diets 1 and 2*

Diet no.	Ground yellow corn	Pure wheat bran	Rolled oats	Alfalfa leaf meal	Desiccated meat meal	North Atlantic fish meal	Dried buttermilk	Ground limestone	Special steamed bonemeal	Anhydrous sodium sulphate	Sodium chloride
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1	39.5	19.3	11.9	4.3	8.0	7.0	5.0	2.5	1.5	0.5	0.5
2	38.3	18.7	11.5	4.2	7.7	6.8	4.9	6.9		.5	.5

TABLE 2.—*Proximate chemical analyses of diets 1 and 2*

Diet no.	Moisture	Crude protein	Ash	Ether extract	Crude fiber	Nitrogen-free extract
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1	11.0	21.0	7.9	4.8	4.1	51.2
2	9.4	21.0	11.7	4.0	3.7	50.2

The estimated vitamin A potency of each of these diets was about 4,000 International units per kilogram of feed. Sherwood and Fraps

(*8*), in their most recent publication on the vitamin A requirements of hens, give a tentative estimate of 3 Sherman-Munsell units of vitamin A potency per gram of total feed as a sufficient quantity for keeping laying hens in good health and production. This estimate is equivalent to approximately 4,200 International units per kilogram of feed. Russell, Taylor, and Chichester (*7*) worked with diets containing 880 to 16,000 International units per kilogram of feed. The records they obtained on seven pens of pullets—

> revealed that the largest quantity of the factor ingested did not increase production, decreased mortality only slightly, and did not improve hatchability as compared with the smallest quantity fed.

Therefore, it is reasonably safe to assume that the diets used in the experiment herein reported had a vitamin A content sufficient to meet at least the minimum requirements of laying hens.

During both years of the experiment the birds in two pens received no vitamin D supplement, those in two other pens received 2 percent, by weight, of cod-liver oil added to the diet, and those in the two remaining pens received ultraviolet irradiation 15 minutes daily, except Sundays, at a distance of 3 feet from the source. A carbon arc, burning special carbons, was used for this irradiation. These carbons contained a quantity of silicon and had iron, aluminum, and nickel in the core. They were designed to produce a high intensity of radiation between 3,400 and 4,000 angstrom units and to give a more even distribution of radiation throughout the entire ultraviolet range than any other type of carbons then available.

The weights of the birds were obtained at the beginning of the experiment and at the end of every 4-week period thereafter. A record was kept of the weight of the feed given to the birds and the weight of the residue left at the end of each 4-week period. Four times a day throughout the experiment the birds in the trap nests were removed and the eggs were collected. The eggs obtained during the last 2 weeks of each of 16 consecutive 6-week periods were weighed. Their contents were removed, and the shells were rinsed with tap water to wash away all adhering albumen. After the shells and membranes had dried thoroughly, they were weighed, and composite samples were prepared for chemical analysis. In order to obtain data on the hatchability of the eggs produced by the birds on the different diets, eggs saved for 10 days were set in the incubator every 6 weeks.

EXPERIMENTAL RESULTS

In general, the results obtained for each pair of pens receiving vitamin D from the same source agreed very closely. Accordingly only the average results of each pair of pens are presented. In the case of the birds receiving ultraviolet irradiation, the agreement was not so good as in the other two cases, but this lack of good agreement is considered in no way to invalidate the conclusions drawn from the data.

A summary of the data is presented in table 3, and the significance of the differences shown in table 3 is given in table 4. The data on live weights of the birds, feed consumption, egg weights, weights of the eggshells, and hatchability of the fertile eggs set were analyzed by appropriate methods of variance analysis. The data on egg production and the total weight of eggs produced per bird were adjusted by covariance analysis for the number of days each bird lived.

TABLE 3.—*Influence of cod-liver oil and ultraviolet irradiations as sources of vitamin D on the egg production, weight of eggs, weight of egg shells, hatchability of eggs, feed consumption, and live weight of Rhode Island Red hens* [1]

Year and source of vitamin D	Birds		Live weight of birds [2]	Feed consumption per bird per period of 4 weeks [3]	Eggs produced per bird [4]	Feed consumption per dozen eggs	Egg weight [5]	Total weight of eggs per bird [4]	Weight of egg-shells with shell membranes [6]	Hatchability of fertile eggs [6]
	Beginning of experiment	End of experiment								
First year (52 weeks):	*Number*	*Number*	*Grams*	*Grams*	*Number*	*Grams*	*Grams*	*Grams*	*Grams*	*Percent*
Cod-liver oil	30	25	2,499±18.0	3,654±53.0	153.9±8.3	3,711	57.2±0.8	8,864±486	4.97±0.08	61.6± 2.5
Ultraviolet irradiation	30	25	2,368±18.0	3,458±53.0	108.5±8.3	4,994	57.0± .9	6,183±486	4.69± .09	48.3± 3.3
None (control group)	30	22	2,210±18.0	3,108±53.0	38.1±8.3	12,626	52.8± .8	2,004±486	3.73± .09	44.0± 6.1
Second year (48 weeks):										
Cod-liver oil	25	21	2,678±27.0	3,376±36.0	108.6±6.6	4,823	59.3± .9	6,477±405	5.28± .13	61.8± 3.5
Ultraviolet irradiation	24	16	2,444±27.0	3,483±36.0	75.4±6.7	7,187	58.8±1.0	4,514±422	4.93± .15	53.6± 4.8
None (control group)	20	17	2,285±27.0	3,118±36.0	6.6±7.3	67,546	54.4±1.0	319±463	3.59± .17	8.3±16.5

[1] Standard errors are used.
[2] Average of the average live weights, per bird, at the beginning of the experiment and at the end of each 4-week period of the year.
[3] Average of the average weights of feed consumed per bird during each 4-week period of the year.
[4] Adjusted by covariance for the number of days each bird lived. The average number of days each bird lived during the first year was 338.7 out of a possible 364, and during the second year was 314.4 out of a possible 336.
[5] Average of the average weights in the case of individual birds.
[6] Weighted average of the percentage hatchability of the eggs of the individual birds

TABLE 4.—*Statistical significance of the differences in egg production, weight of eggs, weight of eggshells, hatchability of eggs, feed consumption, and live weight of Rhode Island Red hens receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D, and those receiving no vitamin D*

Year and source of vitamin D [1] compared	Degree of significance [2] between differences in—						
	Live weight of birds	Feed consumed per period of 4 weeks	Eggs produced per bird	Egg weight	Weight of all eggs produced per bird	Weight of eggshells with shell membranes	Hatchability of fertile eggs
First year (52 weeks):							
Cod-liver oil and ultraviolet irradiation	H	S	H	N	H	S	H
Cod-liver oil and none (control)	H	H	H	H	H	H	S
Ultraviolet irradiation and none (control)	H	H	H	H	H	H	N
Second year (48 weeks):							
Cod-liver oil and ultraviolet irradiation	H	S [3]	H	N	H	N	N
Cod-liver oil and none (control)	H	H	H	H	H	H	H
Ultraviolet irradiation and none (control)	H	H	H	H	H	H	S

[1] Differences are in favor of the first-mentioned source of vitamin D of each comparison.
[2] H=highly significant (odds of at least 99 to 1); S=significant (odds of at least 19 to 1); N=not significant.
[3] Difference is in the favor of ultraviolet irradiation.

For both years of the experiment, the average live weight of the birds receiving cod-liver oil was significantly greater than that of the birds receiving ultraviolet irradiation. Both of these groups had significantly better live weights than did the group receiving no vitamin D supplement.

During the first year the average weight of the feed consumed per bird in the group receiving cod-liver oil was significantly greater than that in the group receiving ultraviolet irradiation, whereas during the second year the reverse was true. Throughout both years the average weight of the feed consumed per bird in the latter group was significantly greater than that in the group receiving no vitamin D.

During the first year the quantity of feed consumed per dozen eggs by the group receiving cod-liver oil was 74.3 percent of that consumed by the group receiving ultraviolet irradiation, and 29.4 percent of that by the group receiving no vitamin D. During the second year the quantity consumed by the first-mentioned group was 67.1 percent of that consumed by the group receiving ultraviolet irradiation and 7.1 percent of that for the group receiving no vitamin D.

The average number of eggs per bird in the group receiving cod-liver oil was significantly greater than that in the group receiving ultraviolet irradiation. The egg production of either group receiving vitamin D was significantly greater than that of the group receiving none.

For both years the eggs from the hens receiving ultraviolet irradiation had about the same average weight as those from the hens receiving cod-liver oil. However, the eggs from the birds receiving no vitamin D averaged approximately 4 to 5 g lighter than those from the other two groups.

Although there was no significant difference between the average weights of the eggs from the birds receiving vitamin D from either of the two sources used in this experiment, nevertheless a highly significant difference existed between the total weights of the eggs produced

per hen from these two groups. During the entire experiment the total weight of the eggs produced per bird in the group receiving ultraviolet irradiation was 69.7 percent of the total weight of the eggs per bird in the group receiving cod-liver oil.

During the first year the eggshells from the birds receiving cod-liver oil were significantly heavier than those from the birds receiving ultraviolet irradiation but during the second year the difference was not significant. The eggshells from the birds receiving no vitamin D were much lighter than those from birds receiving this vitamin.

The agreement between the hatchability results of the first year and those of the second is not good. During the first year there was no significant difference in the hatchability of the eggs from the hens

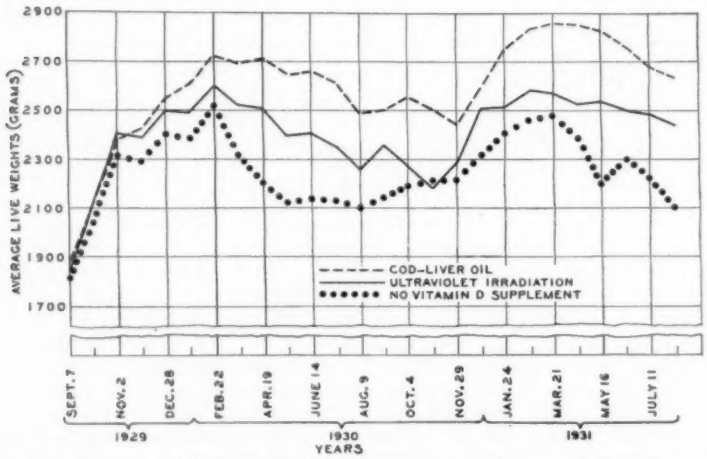

FIGURE 1.— Comparison of the live weights of birds receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D with those of birds receiving no vitamin D supplement.

receiving ultraviolet irradiation and those from hens receiving no vitamin D. On the other hand, the hatchability of eggs from the birds receiving cod-liver oil was significantly greater than that of the eggs from the other two groups. The second year's data show a marked decrease from that of the first year in the hatchability of the eggs from the hens receiving no vitamin D, whereas there was a slight increase in that of the eggs from the hens receiving ultraviolet irradiation.

Data are presented graphically in figures 1 to 6 to show the seasonal fluctuations and the differences among the three groups throughout the experiment. As shown in figure 1, the average live weight per bird in the group receiving ultraviolet irradiation and in the group receiving cod-liver oil were approximately the same during the first 8 weeks of the experiment, but from the eighth week to the end of the experiment the latter group maintained the highest body weight. The seasonal fluctuations were similar in the two groups receiving vitamin D. However, the weight of the birds receiving ultraviolet irradiation dropped more rapidly from February to November than

did that of the birds receiving cod-liver oil and failed to rise any higher the second year than it did the first.

Figure 2 shows that the cumulative feed consumption of the three groups was practically the same for about 16 weeks from the beginning of the experiment. After that the feed consumption of the birds receiving cod-liver oil gradually increased over that of the birds receiving ultraviolet irradiation until there was a difference of 2½ to 3 kg in the middle of the first spring. Thereafter the consumption of the former group was parallel to that of the latter until the following spring, when the difference tended to disappear. On the other hand, the feed consumption of the birds receiving no vitamin D supplement

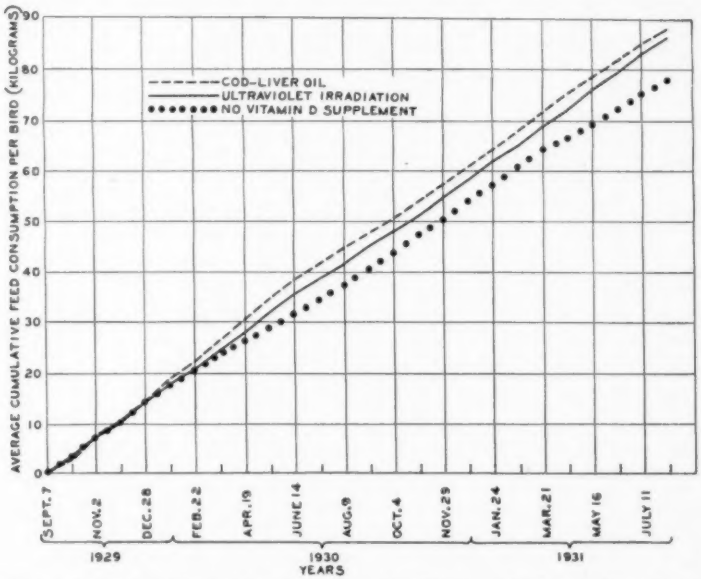

FIGURE 2.—Comparison of the cumulative feed consumption of birds receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D with those receiving no vitamin D supplement.

decreased to about 4½ kg below that of the birds receiving ultraviolet irradiation and was parallel to the latter until March of the second year, when the difference became greater.

Figure 3 shows that the percentage of egg production of the birds receiving cod-liver oil was consistently greater than that of the birds receiving ultraviolet irradiation except during the first 10 weeks of the experiment. The egg production of the hens receiving no vitamin D supplement was much lower than that of the hens receiving either cod-liver oil or ultraviolet irradiation. However, for the first 12 weeks, ended November 30, the former birds maintained as good production as the latter. A sharp decrease in the egg production of the birds receiving ultraviolet irradiation occurred at the same time as that of the birds receiving no vitamin D. However, in the former group, this drop was checked during January, and a gradual upward trend began again. In the latter group, the production decreased to

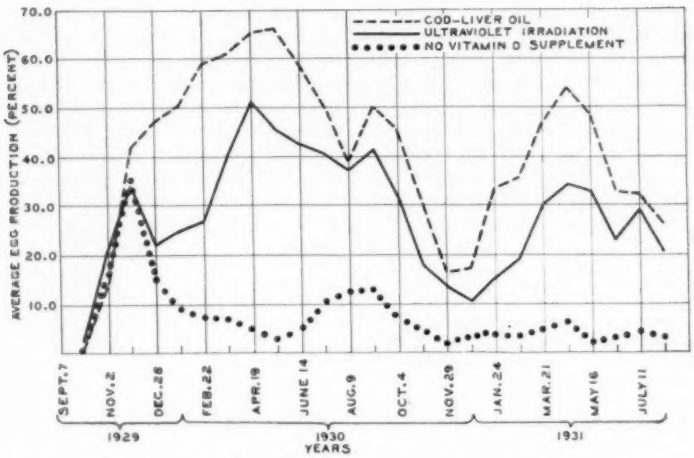

FIGURE 3.— Comparison of the egg production of birds receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D and that of birds receiving no vitamin D supplement.

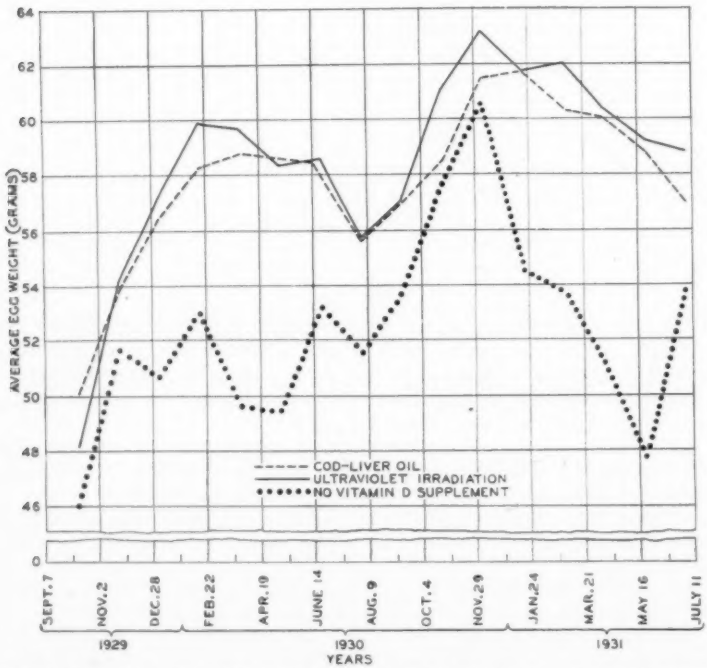

FIGURE 4.—Comparison of the weights of eggs from birds receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D with those from birds receiving no vitamin D supplement.

2.8 percent in May and, except for the slight increase in July and August, it was below 10 percent for the remainder of the experiment. The egg production of the birds receiving cod-liver oil was not decreased in December of the first year as was that of the other two groups, but its rapid upward trend was checked to a slight extent.

It is surprising to note in figure 4 that from early in August to early in December of the second year, the weight of the eggs from the birds receiving no source of vitamin D rose rapidly until it was nearly as high as that of the eggs from the birds receiving cod-liver oil. The eggs from the birds receiving either source of vitamin D were heavier the second year than the first year. Those from the birds receiving ultraviolet irradiation were of greater weight than those from the

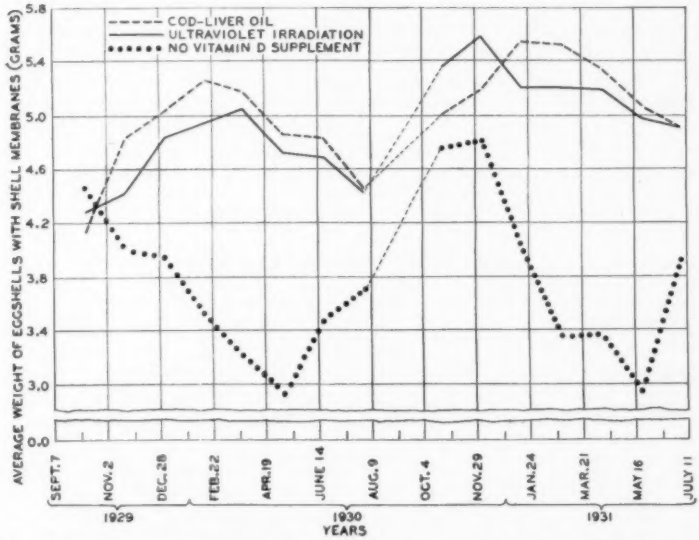

FIGURE 5.—Comparison of the weights of eggshells with shell membranes of eggs from birds receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D with those from birds receiving no vitamin D supplement. Data are not available for the interval between the eleventh and fifteenth periods, as indicated by light dotted lines.

birds receiving cod-liver oil. However, the difference is not statistically significant.

A phenomenon similar to the one in figure 4 also occurs in figure 5. During the early part of December of the second year, the average weight of the eggshells from birds receiving no vitamin D supplement was higher than at any other time, 4.81 g, which is 86.2 percent of the greatest average weight of the eggshells from the birds receiving ultraviolet irradiation and 86.8 percent of those from the birds receiving cod-liver oil. By May, however, the average weight of the eggshells from the hens receiving no vitamin D had dropped to 2.94 g.

Figure 6 shows the ratios of the weight of eggshell to the weight of the whole egg. These ratios, in the case of the birds receiving no vitamin D supplement, show great fluctuations. Like the egg weights and the shell weights plotted in figures 4 and 5, respectively, the ratios

decreased to a low point in the spring of each year and increased to a high level in the late fall. For the other two groups of birds the fluctuations are less. The curve showing the ratios for the birds receiving ultraviolet irradiation are less variable than that for the birds receiving cod-liver oil.

So far as the author knows, nothing has been published heretofore on the effect of vitamin D, from different sources, on the percentage of ash, calcium, and phosphorus in eggshells. The percentage of ash in the shells of eggs from each of the three groups of birds in this ex-

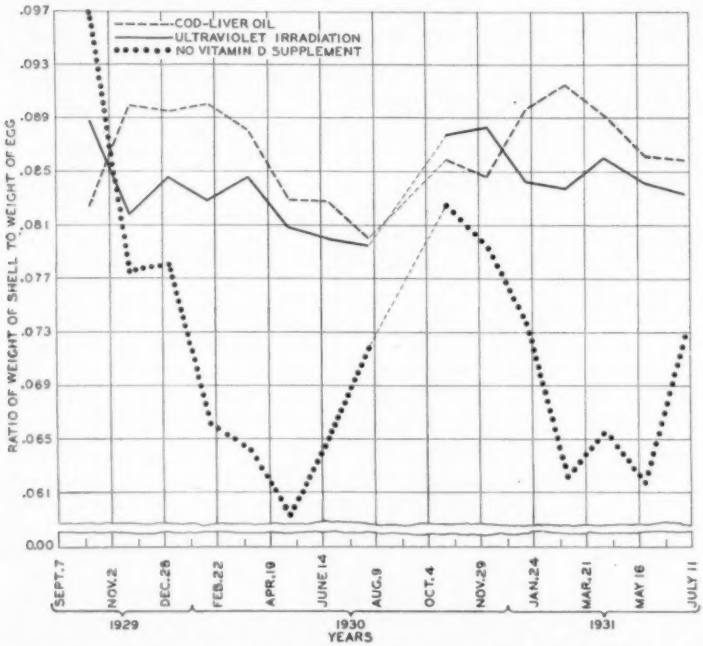

FIGURE 6.—Comparison of the ratios of the weight of eggshell to the weight of the whole egg for birds receiving either cod-liver oil or ultraviolet irradiation as a source of vitamin D with the ratios for the birds receiving no vitamin D supplement. Data are not available for the interval between the eleventh and fifteenth periods, as indicated by light dotted lines.

periment showed minor fluctuations throughout the year, but there was no marked seasonal trend. In the eggshells from the birds receiving no vitamin D supplement the ash was slightly, but not significantly, less than in those from the birds receiving either cod-liver oil or ultraviolet irradiation. The average ash content of all shells, on a dry basis, was 53.33 percent. The percentage of calcium in the eggshells from the birds receiving cod-liver oil and those from the birds receiving ultraviolet irradiation were both 36.63 percent as compared with 36.38 percent in the eggshells from the birds receiving no vitamin D. However, this difference is not statistically significant. The percentage of phosphorus in the eggshells from the birds receiving ultraviolet irradiation and those from the birds receiving no vitamin

D were both 0.158, whereas in the eggshells from the birds receiving cod-liver oil it was only 0.149. In a previous 2-year experiment a similar phenomenon was noted in which there was a difference of the same magnitude.[3]

DISCUSSION

The work herein reported substantiates the conclusion of Hendricks, Lee, and Godfrey that the inclusion of cod-liver oil as a source of vitamin D in the diet of confined laying hens is superior to the administration of ultraviolet irradiation for 15 minutes daily.

The remarkable increase in egg production of the birds receiving no vitamin D supplement during the first 12 weeks of the experiment (fig. 3) indicates that there may have been enough vitamin D stored in the bodies of the birds at the beginning of the experiment to meet their requirements for that length of time. Apparently as a result of this initial increase in egg production at a time when the supply of vitamin D was inadequate, the weight of the eggshells was abruptly reduced from the beginning. The writer is at a loss to explain why the weights of the eggs and of the eggshells fell to their lowest point in the spring of each year, and reached their highest point in the late fall. This phenomenon occurred to a slight extent in the experiment reported by Hendricks and his associates, but not in so marked a manner as in the present experiment.

SUMMARY AND CONCLUSION

Data on live weight, feed consumption, egg production, egg weight, hatchability of fertile eggs, weight of eggshells, and the chemical composition of eggshells were obtained on three groups of Rhode Island Red chickens kept in confinement for 2 years without access to direct sunlight. One group received cod-liver oil fed at a 2-percent level, another received ultraviolet irradiation administered for 15 minutes daily with a carbon arc, and a third received no vitamin D supplement. A definite level of phosphorus and a definite calcium-phosphorus ratio were maintained throughout each year. The same diet was fed for the duration of the experiment, except for a change in the mineral content at the end of the first year. Its vitamin A content was sufficient to meet the minimum requirements of a laying hen.

Cod-liver oil as a source of vitamin D gave better results in egg production, live weight of the hens, total weight of eggs produced per hen, weight of eggshells, and hatchability of fertile eggs, than did ultraviolet irradiation for 15 minutes daily. However, the differences in the results obtained from the two sources of vitamin D on the weight of eggshells and on the hatchability of eggs during the second year were not statistically significant. For maintenance of good egg weight, ultraviolet irradiation was nearly as efficacious as cod-liver oil. More feed was consumed per bird with cod-liver oil than with ultraviolet irradiation, but less feed was required for each dozen eggs produced.

The percentage of ash and calcium in the eggshells was practically the same for both sources of vitamin D. However, the percentage of phosphorus in the eggshells from the birds receiving cod-liver oil

[3] Unpublished data.

was somewhat lower than that in the eggshells from either the birds receiving ultraviolet irradiation or those receiving no vitamin D supplement.

The results thus show conclusively that 2 percent of cod-liver oil added to a good laying diet for strictly confined birds is superior, as a source of vitamin D, to ultraviolet irradiation for 15 minutes daily with a carbon arc. Either source of vitamin D yields better results than an inadequate supply of the vitamin.

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BACTERIAL LEAF SPOT OF BEGONIA [1]

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INTRODUCTION

For some years begonias affected with a bacterial leaf spot have been sent to the United States Department of Agriculture for diagnosis and for advice regarding control or prevention of the disease. The earliest record is a report (without specimens), in 1907 from Nebraska, to the Division of Mycology and Disease Survey, Bureau of Plant Industry, of the occurrence of bacterial trouble on begonia leaves. This was followed by a number of other reports of its occurrence in various States. During the past 4 or 5 years specimens and reports have come in more frequently and from widely separated localities. Considering the length of time the disease has been known, the records contain very little information regarding the causal organism. Losses of from 1 to 50 percent are reported, mostly in young plants. The considerable variation in its occurrence and in the degree of infection is perhaps closely connected with cultural conditions.

REVIEW OF LITERATURE

In 1894 Prillieux and Delacroix (*8*) [2] noted a bacterial stem disease of begonias in France and stated that cultures of the organism resembled *Bacillus pyocyaneus*.

In 1909 Heald and Wolf (*5*) collected diseased begonia leaves in Texas and reported later (*6, pp. 82–83*):

> Begonia. Bacterial leaf-spot (*Bacillus pyrocyanus* [*B. pyocyaneus*] P. and D. (?).— * * * This is probably the same disease that occurs in France, although it was not observed to attack the stems but only the leaves. * * * No cultural work with the organism was attempted.

Begonia leaf specimens of the Heald and Wolf collection of 1909 are now in the mycological collections of the Bureau of Plant Industry (no. 1411, bacterial leaf spot, *Bacillus pyrocyanus* [*B. pyocyaneus*] on begonia). These specimens and a photograph of an infected leaf in Heald and Wolf's publication (*6*) show lesions entirely similar to the leaf spot described in the present paper.

O. C. Boyd [3] reported this bacterial leaf spot of begonia from Georgia in 1928 and sent typical specimens to the Division of Mycology and Disease Survey. R. P. White reported in 1928 that the variety Melior is very susceptible and the variety Peerless is resistant to the bacterial leaf spot of begonias. In 1929 he reported that there was a yellow halo about each spot. The writer in 1930 reported that the colonies of the causal bacteria are yellow. Numerous reports of its

[1] Received for publication Aug. 1, 1936; issued May 1937.

[2] Reference is made by number (italic) to Literature Cited, p. 589.

[3] The reports in this paragraph relate to card records on file in the Division of Mycology and Disease Survey, Bureau of Plant Industry, U. S. Department of Agriculture.

occurrence came in before 1930, but unfortunately specimens were seldom sent.

In Europe, Buchwald (*2*) in 1929 and again (*3*) in 1933 reported a bacterial disease of begonias. Fotsch (*4*) in 1933 mentions the disease. In each report the European disease is described as a vascular infection. Leaf veins are blackened, leaves wilt, stems rot and fall over. Buchwald (*3*) states that the symptoms are similar to those caused by *Bacterium campestre* in cabbage. He named the causal organism *Bact. begoniae*.

In 1935 Wieringa (*10*) reported more fully a wilt and stem rot disease of begonias. His descriptions of the symptoms and the causal bacterium suggest *Bacterium campestre*. Wieringa named the organism *Phytomonas flava begoniae*. It is an interesting fact that the records of bacterial diseases of begonias in Europe from 1894 to 1935 are of vascular infections with resulting leaf wilt, vein blackening, and stem rot, whereas in the United States all the records are of leaf spots with no indication of vascular disease. Allowing for possible errors in description or technique, the bacterium of the European vascular disease is very similar in character to the bacterium causing leaf spot disease in the United States. It is possible but not likely that the same or a very similar organism affects vascular tissue under some conditions and only leaf mesophyll under other conditions.

The bacteria on which the present study is based were isolated from begonia leaves (var. Lloydii) collected in Oregon in June 1934 by F. P. McWhorter, plant pathologist, of the Oregon State College, and from leaves inoculated and infected with this Oregon strain.

DESCRIPTION OF THE DISEASE

The spots are usually well isolated and well distributed over the leaves (fig. 1). Sometimes they are more numerous along the leaf margin, but are never observed on the petioles or veins. They are occasionally found on the leaf bracts.

Infections are first visible as tiny, clear specks on the lower leaf surface. These enlarge into more or less circular, translucent, pale-green or colorless spots that later have brown, opaque centers with translucent, yellow halos. The size varies from 1 to 8 mm in diameter, averaging 2 to 5 mm. The bacteria probably enter through the stomata, which are fairly numerous on the lower surface of the leaf, fewer on the leaf bracts, very rare on the flower parts, and absent from the upper leaf surfaces, veins, petioles, peduncles, and stems. Sections through infected areas show that the destruction of tissue and the multiplication of the bacteria begin in the lower layers of cells, later spreading to the palisade cells and upper epidermis. With conditions favorable for the parasite the spots enlarge and coalesce, sometimes forming rather large areas of affected tissue. If considerable moisture is present these areas undergo soft rot. In no case has there been any evidence of vascular infection, of wilt or stem rot, or of entire destruction of the plants. However, the attacked plants become unsightly and worthless for sale, because even the moderately infected leaves fall and the less infected ones are imperfect. The plants continue to grow, but parts of the stem remain bare and the new leaves are often small. The new leaf growth is apparently healthy, but in many cases a trace of infection is present in tiny, inconspicuous brown

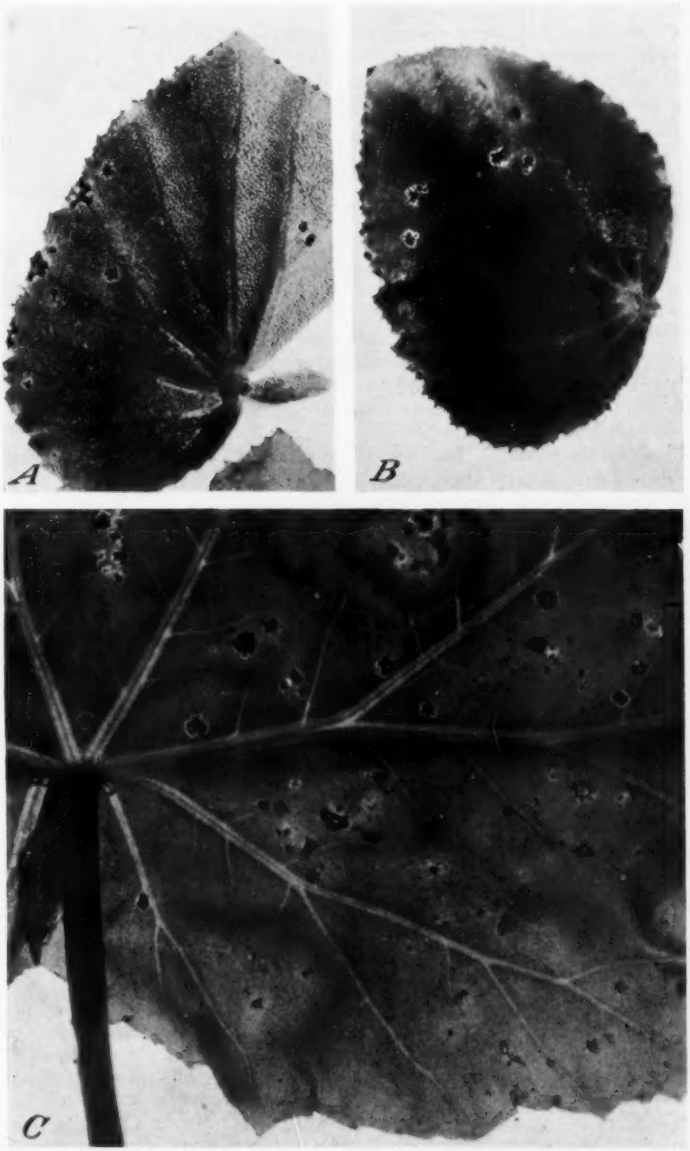

FIGURE 1.—*A*, *Begonia semperflorens*, 10 days after inoculation; *B*, natural infection on *B. semperflorens*; *C*, *Begonia*, hort. var. Jessie, 16 days after inoculation. All × about 2.

spots from which a general infection spreads whenever conditions are again favorable for the parasite.

Warm, moist, poorly ventilated, and crowded conditions favor the development of the disease. Adequate spacing, with regulated temperature and moisture, is usually effective in preventing or in curing the disease. Rapid forcing should be avoided, and spotted leaves should be removed or destroyed.

THE CAUSAL ORGANISM

ISOLATION AND INOCULATION

Pure cultures of the bacteria are easily isolated from recent lesions. Other types of bacteria are sometimes found in old lesions and in the soft rot encouraged by moisture. The organism has been isolated repeatedly from natural infections and from those resulting from inoculations. All of the available species and varieties of begonia—*rex, lucerna, semperflorens*, Chatelaine, *maculata, ricinifolia*, "Lorraine", Jessie, and one unknown variety—have always developed the typical leaf spots after the plants have been inoculated by spraying them with water containing the bacteria.

The stems, petioles, leaf veins, and flowers appear to be immune. Repeated and heavy inoculations by various methods failed to give even a trace of infection in these tissues.

Because the organism causing this leaf spot of begonia so closely resembles in its morphological and cultural characters the group of yellow bacteria of which *Bacterium campestre* is perhaps the best known representative, it was necessary to test its pathogenicity on various hosts. Cabbage, rutabaga, bean, radish, and horseradish plants were inoculated in parallel tests with begonias. The tests were repeated a number of times under different conditions of moisture and temperature. No infection developed on any of the plants except the begonias, which invariably became typically infected.

Geraniums (*Pelargonium* spp.) are very slightly susceptible to the begonia pathogen. In one of several inoculation tests, small, circular, translucent spots appeared on the leaves. Common garden varieties of geranium (*P. hortorum* Bailey) and the Lady Washington (*P. domesticum* Bailey) were used in these tests.

Bacterium pelargoni Brown (*1*) and *Bact. erodii* Lewis (*7*) cause definite leaf spotting of geraniums. These organisms are unlike the begonia organism.

MORPHOLOGY

The bacteria are slender rods with rounded ends. Stained with carbol-fuchsin, single rods are 0.9μ to 1.8μ long and 0.3μ to 0.4μ wide. Paired rods are frequent in most culture media. Chains occur in beef broth and in beef broth plus 2-percent sodium chloride. No definite capsules were demonstrated. There is a slimy substance in practically all cultures, and this sometimes suggests a capsule, as it stains less deeply than the rod. No spores or involution forms have been observed. The bacteria are motile. There is a single, polar flagellum three to four times the length of the rod and distinctly wavy. The bacteria are Gram-negative and are not acidfast. They stain readily with the usual aniline stains.

CULTURAL CHARACTERS

On beef-peptone agar [4] plates inoculated directly from a leaf spot or from a culture, colonies appear in 2 days, and in 4 days well-isolated colonies are 4 mm in diameter. The colonies are circular, smooth, flat, with margins entire and translucent. The centers are nearly or quite opaque, but with age the whole colony becomes transparent. The color is "massicot yellow." [5] Submerged colonies are opaque, oval to broad spindle-shaped.

Beef-agar slants have a smooth, thin, translucent, later transparent growth. Sometimes there is a small opaque area of growth at the base of the slant.

In beef-agar stabs the growth is thicker but slow in covering the surface, and after several weeks small secondary centers of growth appear. There is no growth in the stab.

Growth on beef-extract agar is similar to but considerably less than that on the beef-infusion agar.

On all the solid beef media the growth is usually slightly viscid.

In beef-peptone broth growth begins at the surface as a moderate clouding which increases to form irregular rims and fairly heavy, discontinuous, yellow pellicles. This surface growth breaks into irregular pieces and falls if even slightly disturbed. Pseudozoogloeae are often present. There is a slight translucent yellow sediment. The clouding persists for 3 to 4 months and the medium becomes viscid, extremely so in the lower half of the culture. No green pigment was formed in any culture.

Thickly sown beef-gelatin plates at 20° C. are completely liquefied in 3 days; thinly sown plates liquefy in 8 days. The opaque, granular, yellow colonies remain intact and floating in the clear liquid. Gelatin tubes at 10° to 12° are entirely liquefied in 8 to 10 weeks; the medium is clear, without surface growth, but with yellow sediment at the base of the tube.

On potato-dextrose agar of pH 5.6 to 5.8 the growth is rapid and abundant. The surface becomes covered with thick, soft, opaque growth. The color is at first "honey yellow," later "tawny olive" or "isabella color."

Growth on potato cylinders is luxuriant, thick, smooth, shining, and deeper yellow ("old gold" or "buffy citrine") than on beef media. Cultures 8 weeks old are "olive brown."

In Cohn's solution there is no growth. Uschinsky's solution clouds slowly. In 2 weeks there is a definite surface growth that slowly increases to form yellow ("Naples" to "mustard") rims and pellicles. There is a slight, pale-yellow sediment. The medium between the surface growth and the sediment is a clear, pale yellow.

Growth is good in nitrate broth, but there is no reduction of nitrates to nitrites in 7-, 10-, or 16-day cultures. *Bacillus phytophthorus* Appel grown in the same medium and tested by the same methods gave a positive test for nitrate reduction (tests were made with the α-naphthylamine sulphanilic acid method).

In beef broth plus 2-percent sodium chloride, growth is considerably retarded and limited to a thin surface clouding and thin rims (the

[4] Unless otherwise stated, all beef media were made with beef infusion and had a pH value of 6.8 to 7.0.
[5] The color readings given in quotations are based on Ridgway's Color Standards (9).

yellow rim growth is very viscid). In 3-percent sodium chloride the growth is even less, and in 4-percent the growth is entirely inhibited.

Very good growth developed in a 1-percent tryptophan solution, but tests made on the fourth and on the tenth day gave no evidence of indol (tests were made with paradimethylaminobenzaldehyde and potassium persulphate).

There is definite evidence of hydrogen sulphide in potato-dextrose agar, potato cylinders, milk, and beef cultures.

Ammonia in small amounts is produced in most culture media.

Milk cultures quickly show a layer of clear whey at the surface. In 8 to 10 days the milk is colorless and transparent except for the yellow surface film and the scanty translucent yellow sediment. The liquid becomes pale amber or pale orange and the translucent sediment increases until it fills the lower half of the tube. Tyrosine crystals were abundant in some cultures and rather scanty in others.

Litmus in milk is completely reduced in 8 to 10 days. The color begins to return about the fifteenth day. Old cultures are pale wine red.

Fermentation and acid reaction are prompt and strong in saccharose, slightly less in dextrose, slow and weak in lactose, still slower in mannite and glycerin. Some strains failed to grow in the glycerin medium. The medium used for these tests was a peptone-free, synthetic agar with brom-cresol purple as indicator and 1 percent of the carbohydrate to be tested. No gas was produced and no clouding occurred in the closed arm of fermentation tubes containing the same medium without agar.

The optimum pH value for growth in beef media is 6.8 to 7.0, and 5.0 and 8.8 are the limits for growth.

The diastatic action was tested in plate cultures of beef-extract agar plus 0.2 percent starch. Surface streak inoculations produced vigorous and abundant growth. Tests with an iodine solution on the third day showed entire starch reduction in a band 10 to 11 mm wide on either side of the bacterial growth. On the ninth day this reduction was 30 to 35 mm wide. Parallel plates, with brom-cresol purple as an indicator, showed that no acid production accompanied the starch reduction.

The optimum temperature for growth is about 28° C. Cultures in liquid and on solid media produced under the same conditions equal growth at 27°, 28°, and 29°. The maximum temperature is 37° or slightly higher. Liquid media at 37° produced no growth, but potato-dextrose agar slants showed visible growth in 3 days at 36° and in 6 days at 37°. The minimum is 8° or lower. The thermal death point is about 50°. Cultures on solid media produced visible growth more quickly and also endured greater extremes of temperature than liquid cultures. Well-developed cultures that had been frozen solid for 10 days were only slightly reduced in vitality. Freshly inoculated cultures were frozen (−3°) for 25 days, then removed to room temperature. Growth was slow in starting, but in 10 days after thawing it was definite and typical. Beef-bouillon and potato-dextrose agar cultures were alive and only slightly reduced in vigor after being frozen for 5 months.

Cultures at room temperature and at 15° C. remained alive for 12 months or more but were reduced in vigor after 8 to 10 months. When dried on cover glasses, the vitality of the bacteria was good for 40 days, but afterwards many tests failed; the last positive test was after 56 days of desiccation. Exposure of freshly inoculated plates to direct sunlight for 20 minutes killed all the bacteria. Exposure for 15 minutes caused a reduction of 98 to 99 percent; 10 minutes, 50 percent; and exposure even for 5 minutes caused a noticeable reduction in numbers.

TECHNICAL DESCRIPTION

Bacterium flavozonatum n. sp.

Slender rods, rounded at ends, single and in pairs. Average size 0.9μ to 1.8μ by 0.3μ to 0.4μ. Motile; single, wavy polar flagellum, three to four times the length of the rod. Capsules absent, or very indefinite. No spores nor involution forms. Aerobic. Growth yellow on beef agar. Gelatin liquefied. Ammonia and hydrogen sulphide produced. Nitrates not reduced. Good growth in Uschinsky's solution. No growth in Cohn's solution. Milk rapidly cleared. Starch hydrolyzed. Maximum temperature 37° C., minimum below 8°, optimum about 28°; thermal death point about 50°. Resists drying 56 or more days. Produces acid but no gas from dextrose, saccharose, lactose, mannite, and glycerin. Gram-negative. Not acidfast. Stains readily with aniline dyes. Pathogenic to begonias, producing leaf spots and causing defoliation.

Specimens of this disease on begonia leaves have been deposited in the mycological collections of the Bureau of Plant Industry.

SUMMARY

A bacterial leaf spot of cultivated begonia that causes defoliation in severe attacks and unsightly plants and leaves in lighter attacks occurs widely in the United States.

The causal organism has been isolated and its pathogenicity for begonias proved. Because the organism resembled *Bacterium campestre*, inoculations were made but no infections were secured on cabbage, rutabaga, radish, and horseradish. On geranium leaves a very slight infection was sometimes produced.

Methods of control and prevention are suggested.

The name *Bacterium flavozonatum* is proposed for the organism causing this begonia disease.

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A LEAF SPOT DISEASE OF RED AND WHITE CLOVERS [1]

By OLIVER F. SMITH [2]

Agent, Division of Forage Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture

INTRODUCTION

In a number of pastures near Madison, Wis., the leaf spot disease herein described was first observed on red and white clovers, *Trifolium pratense* L. and *T. repens* L., in August 1935.[3] In many instances the disease occurred on the lower leaves in a dense growth of vegetation, but it was also found on the leaves of white clover in closely grazed pastures. The damage caused was slight, however, for relatively few leaves were injured, and the fungus was not found attacking other parts of the plants. A fungus that was readily isolated from characteristic lesions has proved to be pathogenic on leaves of red and white clovers as well as on a number of other leguminous plants. As attempts to induce the production of spores in culture have proved unsuccessful, the taxonomic position of the organism has not been determined. So far as known, this organism has not previously been described; inasmuch as the lesions on red clover may easily be mistaken for those produced by *Macrosporium sarciniforme* Cav., a brief description of the disease and of the fungus that causes it is presented herein.

DESCRIPTION OF THE DISEASE

Lesions produced by the fungus are limited to the leaf-blade tissue. Under field conditions they consist of dark-brown necrotic areas which often become irregularly shaped owing to the coalescing of adjacent lesions. Concentric zones are formed around each point of infection (fig. 1), making the lesions appear similar to those produced by *Macrosporium sarciniforme* on red clover. However, a careful examination of affected leaves with the aid of a hand lens or an Ultrapak microscope reveals coarse strands of aerial mycelium produced by this fungus during its spread over the surface of the leaf. This serves to distinguish it from *M. sarciniforme* on red clover. Under conditions of artificial inoculation where pieces of agar containing mycelium are placed on the leaves and the plants are maintained at a high humidity, the fungus grows out from the agar and produces small brown necrotic lesions at the points of infection within a period of 16 to 20 hours after inoculation. While these initial lesions are increasing in size the aerial strands of mycelium spread above the surface of the leaf, attacking it at other places and producing new points of infection (fig. 2). This

[1] Received for publication Aug. 7, 1936; issued May, 1937. Joint contribution of the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture and the Wisconsin Agricultural Experiment Station.

[2] The writer wishes to express his appreciation to Eugene Herrling, assistant in plant pathology, Wisconsin Agricultural Experiment Station, for preparing the photomicrographs.

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procedure continues, and as the lesions increase in size and number they coalesce and kill an entire leaflet in 48 hours after inoculation.

DESCRIPTION OF THE FUNGUS

In culture, the mycelium is hyaline when young but soon becomes dark green and finally dark brown or black with age. It is composed of large, branching, septate filaments filled with granular protoplasm but with numerous vacuoles in the older portions. Cells of the larger filaments average about 16μ in width although many of them are short and almost round, having a diameter of 28μ to 30μ. In shape these short cells appear much like chlamydospores, but their walls are not thickened and the cell contents do not appear different from those of other cells of the mycelium. In some instances the fungus produces numerous clusters of short cells in the media, which

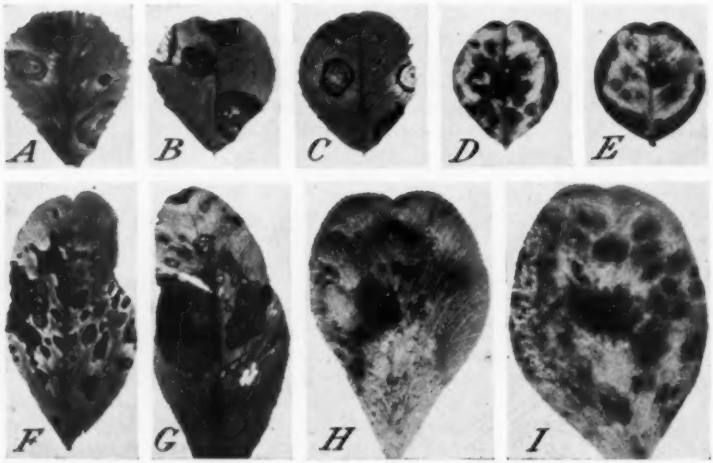

FIGURE 1.—Leaf spot disease on red and white clovers: *A*, *B*, and *C*, Natural infection on leaves of white clover collected in the field; *D* and *E*, leaves of white clover artificially inoculated in the greenhouse; *F* and *G*, natural infection on leaves of red clover collected in the field; *H* and *I*, leaves of red clover artificially inoculated in the greenhouse. The dark material in the center of leaves *D*, *E*, *H*, and *I* is the inoculum from which the fungus has grown out and infected the leaves. About natural size.

resemble conidia in grapelike clusters. These structures occur most frequently at temperatures of 24° and 28° C. and appear microscopically as young sclerotia, but they have never developed into such bodies. De Bary [4] describes similar structures for species of *Sclerotinia*.

On soil-extract agar and potato-dextrose agar, the fungus forms considerable aerial mycelium and a layer of interwoven mycelium over the surface of the substrate. Growth on oatmeal agar is similar to that on potato-dextrose and soil-extract agars except that less aerial mycelium is produced on the former. Neither aerial mycelium nor the membranous layer of interwoven mycelium is formed on cornmeal agar, but the fungus penetrates more deeply into this medium than into those mentioned above.

[4] BARY, A. DE. COMPARATIVE MORPHOLOGY AND BIOLOGY OF THE FUNGI, MYCETOZOA, AND BACTERIA. Authorized Engl. transl. by H. E. F. Garnsey, rev. by I. B. Balfour. 525 pp., illus. Oxford. 1887.

PATHOLOGICAL HISTOLOGY

When ends of branches of aerial mycelium, growing above the surface of inoculated leaves in an atmosphere of high humidity, come in contact with the surface of the leaf, they form groups of short diverging hyphae which apply themselves closely to the surface (fig. 3, *A*, *B*, and

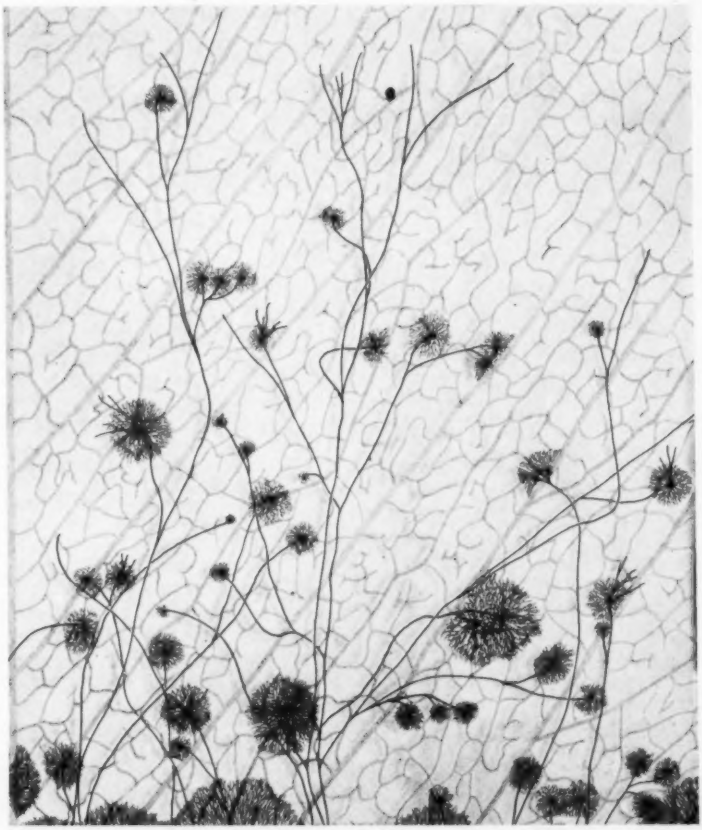

FIGURE 2.—Semidiagrammatic sketch of the leaf spot fungus on a leaf of red clover, showing the manner in which it spreads above the surface of the leaf as strands of aerial mycelium and penetrates into the interior at points of contact between hyphal tips and the leaf surface. Aerial mycelium is represented by dark solid lines, whereas the mycelium within the leaf tissue is represented by the dark stippled areas. About × 18.

C) and serve as organs of attachment. From some of these organs of attachment hyphae continue to grow above the surface of the leaf as aerial mycelium. They do not ordinarily extend far, however, before their ends touch the surface of the leaf and produce additional groups of short diverging hyphae. Similar organs of attachment have been described by Smith [5] for a fungus attacking lettuce grown

[5] SMITH, R. E. BOTRYTIS AND SCLEROTINIA: THEIR RELATION TO CERTAIN PLANT DISEASES AND TO EACH OTHER. Bot. Gaz. 29: 369–407. illus. 1900.

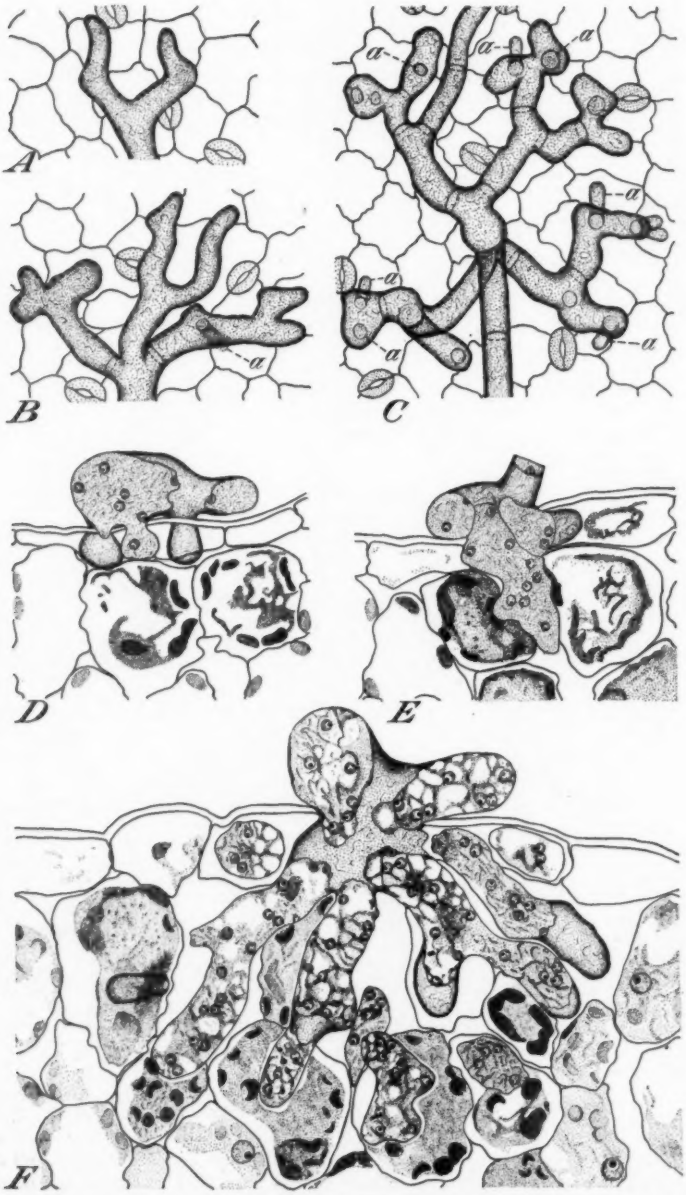

FIGURE 3.—*A*, *B*, and *C*, Camera-lucida sketches of progressive stages in the development of an organ of attachment at a point of contact between a hyphal tip and the surface of a leaf. Young penetration hyphae are shown in *B* and *C* at *a*. About × 260. *D*, *E*, and *F*, Camera-lucida sketches of early stages of penetration and spread of the fungus in the leaf tissue, showing the disorganization of the contents of the host cells in advance of the invading hyphae. About × 660.

in the greenhouse. He states, however, regarding that fungus: "Sclerotia are formed in considerable abundance on affected plants * * *." He states further: "It is also usual to find an abundant, white, woolly, mycelium proceeding from affected plants to the surrounding soil * * *." Neither sclerotia nor white woolly mycelia have been produced by the fungus described herein.

Entrance into the host tissue takes place directly through the epidermis at several points along the short hyphae that are in contact with the leaf surface. Penetration hyphae enter either directly through the epidermal cells or between them but have never been observed to enter through stomata. After entering the epidermal cells, the infection hyphae enlarge to form vesicles similar to those described by Boyle [6] for *Sclerotinia libertiana*. While the infection hyphae are still confined to the epidermal cells, the palisade cells of the host show signs of being chemically altered, as their contents become darkly staining masses of granular material (fig. 3, *D*).

After penetration, the fungus spreads intercellularly and intracellularly through the leaf tissue, killing the host cells in advance of its progress, as the cell contents are disorganized and impoverished some distance beyond the limits of an invading hypha (fig. 4, *B*). Hyphae have been observed to have entered epidermal cells from the inner side and after traversing a number of them to have returned to the region of mesophyll and spongy parenchyma. The fungus is, however, unable to penetrate through the vascular bundles, at least during the early stages of infection, owing to the presence of a comparatively wide sheath of sclerenchymatous cells which almost completely surround the vascular strands.

Penetration and subsequent advance of the fungus through the leaf tissue is very rapid. In leaves that were cleared and stained 24 hours after inoculation, the fungus had advanced through the tissue a maximum distance of 700μ from points of infection.

As the fungus spreads through the interior of the leaf, hyphae grow out through the epidermis on both upper and lower surfaces. Growth of the mycelium to the surface is usually through the stomata but may be either directly through or between the epidermal cells. In this manner aerial mycelium that has collapsed during periods of relatively low atmospheric humidity is replaced. Such mycelium either attacks the leaf at other points or spreads to nearby leaves. Strands of hyphae have been found to have traveled through the air a distance of nearly a half inch from one leaf to another. When several leaves are in close proximity in an inoculation chamber they become so interconnected with hyphae that they are sometimes torn when an attempt is made to pull them apart.

[6] BOYLE, C. STUDIES IN THE PHYSIOLOGY OF PARASITISM. VI. INFECTION BY SCLEROTINIA LIBERTIANA. Ann. Bot. [London] 35: [337]-347, illus. 1921.

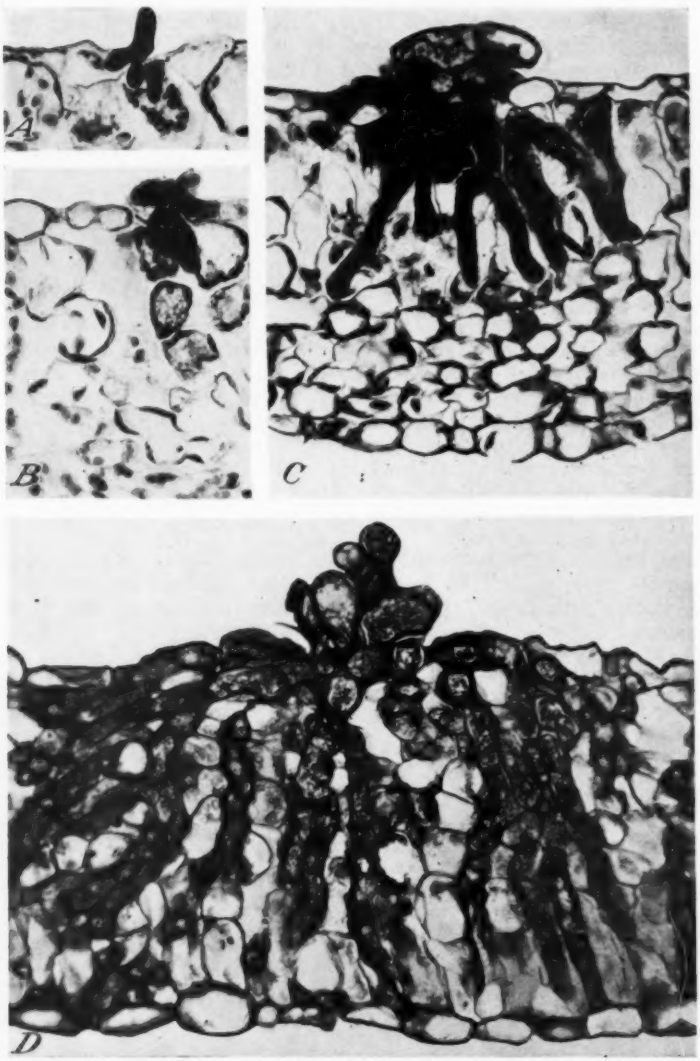

FIGURE 4.—*A*, *B*, *C*, and *D*, Photomicrographs of progressive stages in the penetration and growth of the leaf spot fungus through red clover leaf tissue. Note the killing and impoverishment of the host cells in advance of the infecting hyphae, particularly in *A* and *B*. All the above stages of infection were obtained from leaves 20 hours after inoculation. About × 375.

ENVIRONMENTAL RELATIONS OF FUNGUS

EFFECT OF TEMPERATURE ON GROWTH

In order to determine the optimum temperatures for the growth of this organism, cultures were grown in Petri dishes on potato-dextrose agar in incubators maintained at various temperatures, ranging by 4° intervals from 4° to 36° C. A small piece of agar containing mycelium was placed in the center of each Petri dish, and the diameters of the colonies were measured in millimeters after 120 hours. When clusters of short cells were produced in the media, as described on page 2, the radial growth of the fungus was considerably retarded. The diameters of such colonies were not included in the data on growth, as this phenomenon occurred only at the higher temperatures and such measurements would not be comparable with those of colonies grown at the lower temperatures where this condition did not occur.

The results (table 1) show that the greatest average width of colonies was produced at 24° and 28°.

EFFECT OF TEMPERATURE ON PATHOGENICITY

In order to determine the effect of temperature on pathogenicity, excised leaflets of red clover were inoculated with small pieces of agar containing mycelium and were floated on a 5-percent sucrose solution in Petri dishes that were placed in incubators maintained at various temperatures, ranging by 4° intervals from 4° to 36° C. An uninoculated leaflet was always placed in the dish beside those inoculated. Results were recorded 48 hours after the leaves were inoculated, when, at the optimum temperatures of infection, the fungus had overrun and killed the inoculated leaflets. In some instances the mycelium had extended beyond the edge of the leaflet on the surface of the solution a distance of 2 to 5 mm. At the lower temperatures (4°, 8°, and 12°) no lesions were produced at the end of 48 hours although the fungus had grown a short distance over the surface of the host. With increased time, however, lesions were produced at these temperatures. No growth occurred at a temperature of 36°. The results (table 1) show that a practically equal and maximum amount of infection occurred at 24° and 28°.

TABLE 1.—*Mean diameter* [1] *of colony growth of leaf spot fungus on potato-dextrose agar in 120 hours and mean amount of infection* [1] *on leaves of red clover in 48 hours, at different temperatures*

Temperature (°C.)	Mean diameter [2]	Mean infection [3]	Temperature (°C.)	Mean diameter [2]	Mean infection [3]	Temperature (°C.)	Mean diameter [2]	Mean infection [3]
	Mm			*Mm*			*Mm*	
4	4.3	0	16	38.8	2.5	28	81.1	9.7
8	13.3	0	20	59.5	5.5	32	23.0	7.0
12	26.1	0	24	83.0	9.6	36	0	0

[1] 8 replications at each temperature.
[2] Standard error of difference=1.48.
[3] Based on scale from 0 (=no infection) to 10 (=lethal infection). Standard error of difference=0.325.

RELATION OF HUMIDITY TO GROWTH AND INFECTION

Hyphae of this fungus are very sensitive to atmospheric humidity. When leaves are inoculated and placed in an atmosphere of about 60 to 70 percent relative humidity, the agar dries and the fungus is killed before it can grow out from the medium and infect the leaves. If mycelium is allowed to grow over the surface of a leaf, or on agar, in an atmosphere of high humidity (95 to 100 percent) and is then exposed to an atmosphere of about 70 percent relative humidity, the aerial mycelium collapses almost immediately. When leaves are inoculated and placed in an atmosphere of high humidity until the fungus has become established within the leaf tissue and are then placed in an atmosphere of low humidity, aerial mycelium is killed and the advance of the fungus is limited to growth within the leaf tissue until a high humidity again permits the growth of aerial mycelium.

INFECTION EXPERIMENTS

Tests were made of the susceptibility of 11 species and 1 variety of plants in 6 genera by placing pieces of agar containing mycelium on the leaves and maintaining the plants at a high humidity. After 48 hours the amount of infection on the leaves was estimated on a scale of 0 to 10, where 0 represents no infection and 10 represents the amount of infection produced on leaves of red clover during the same period. The results are given in the following tabulation:

Host:	*Relative amount of infection* [1]
Trifolium pratense L.	10
T. repens L.	10
T. repens giganteum Lagr.-Foss.	10
T. incarnatum L.	9
T. hybridum L.	10
Melilotus alba Desr.	7
M. officinalis Lam.	7
M. indica All.	9
Medicago sativa L.	8
Vicia faba L.	0
Phaseolus vulgaris L.	0
Brassica oleracea L.	0

[1] 0=no infection; 10=amount of infection produced on leaves of red clover in 48 hours.

Red and white clover plants were inoculated on the petioles, stems, crowns, and hypocotyls to determine the ability of the fungus to attack parts other than the leaves. For root inoculations, plants of red clover were grown in boxes specially constructed so one side could be removed and the inoculum applied directly to the young roots with very little disturbance of the soil around them. In none of these tests did the fungus parasitize any of the plant tissues, although the mycelium grew over the surface of the inoculated parts. In another experiment, seeds of red clover were planted in soil that was heavily infested with the fungus, but the seedlings grew as well as those planted in clean soil. From these results it appears that the fungus is unable to attack any part of the plant other than the leaves.

SUMMARY

A fungus that has been found attacking the leaves of *Trifolium pratense* and *T. repens* is described. It has also proved to be pathogenic on leaves of *T. hybridum*, *T. incarnatum*, *T. repens* var. *giganteum*, *Melilotus alba*, *M. officinalis*, *M. indica*, and *Medicago sativa*.

Lesions are limited to the leaf-blade tissue. Under field conditions they consist of dark-brown, often irregularly shaped, necrotic areas. Concentric zones are formed around each point of infection, making the lesions appear similar to those produced by *Macrosporium sarciniforme* on red clover.

Under conditions of high humidity, aerial mycelium grows above the surface of inoculated leaves and at each point of contact between a hyphal tip and the leaf forms a group of short diverging hyphae, which apply themselves closely to the surface and serve as organs of attachment.

Penetration takes place directly through the epidermis at several points beneath these organs of attachment. The mycelium travels intercellularly and intracellularly through the leaf tissue, killing the host cells in advance of its progress.

Temperatures of 24° and 28° C. are most favorable for growth of the fungus on potato-dextrose agar and for the infection of red clover leaves.

As the fungus has produced no spores or sclerotia, either on the host or in culture, its taxonomic position has not been determined.

SELENIUM ABSORPTION BY CROP PLANTS AS RELATED TO THEIR SULPHUR REQUIREMENT [1]

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INTRODUCTION

Observations of marked differences in the selenium-absorbing capacity of plants indigenous to the naturally seleniferous soil areas (*4*) [3] were followed by the discovery that crop plants grown on an artificially selenized soil also differ widely in selenium content (*10*) and that the variations seem to parallel the known sulphur requirements of the different crops. With the purpose of establishing this apparent correlation on a quantitative basis, various crops representing plant families known to differ with respect to their sulphur-absorbing capacity were grown in uniformly selenized soil in the greenhouse and sampled at intervals for determination of their sulphur and selenium content. The results of the analyses are reported in the present paper.

METHODS

Representative crops were grown in separated plots in greenhouse benches containing Keyport clay loam, untreated with respect to sulphur.[4] Into each plot was mixed an aliquot of a stock solution of sodium selenate ($Na_2SeO_4.10\ H_2O$) supplying 4 p. m. m. of selenium by weight of air-dry soil. For two supplementary experiments, 3 and 5 p. p. m., respectively, were used. These concentrations are not toxic to plants in this soil (*10*).

By using varieties [5] not too specific in their temperature requirements and by varying the temperature in the greenhouse in accordance with the growth stages of the plants, it was possible to grow high- and low-temperature crops in the same house, although not all gave normal yields. Thus an initially low temperature of about 15° C., permitting a good start for such crops as wheat, was gradually raised until even corn and sorgo were able to grow and produce seed. Unfortunately, not all of the crops produced seed, and some, viz, onions, alfalfa, millet, and kafir, grew too slowly to supply enough material for sampling at the first two cuttings.

Plants were cut for analysis at three different times during their growth, at the ages of 1, 2, and 3 months, respectively, and dried (24 hours) in a ventilated oven at 60° C.

[1] Received for publication Oct. 8, 1936; issued May, 1937.

[2] The selenium analyses reported herein were made by R. B. Deemer and R. F. Gardiner, under the direction of E. C. Shorey and F. R. Dawson, all of the Division of Soil Fertility Investigations, Bureau of Plant Industry. The sulphur analyses were made by J. B. Martin, of the same Division. The writer is also indebted to C. G. Colcord, of the Division of Cereal Crops and Diseases, for statistical calculations.

[3] Reference is made by number (italic) to Literature Cited, p. 608.

[4] The soil had been analyzed for sulphur previously and found to contain about 0.06 percent, of which 0.005 percent was water-soluble.

[5] Bison flax, Grimm alfalfa, Hard Federation wheat, Abruzzi rye, German millet, Blackhull kafir, Black Amber sorgo, Virginia soybean, and Golden Bantam corn.

The selenium content of the dried and ground tissues was determined by the method for soils described by Robinson et al. (*13*), modified to include a preliminary low-temperature digestion with bromine. From subsequent studies of procedures used for determining selenium in plant materials it appears that the method used here may give low values (*14*), but since the study is one involving relationships rather than absolute values, the conclusions are not affected. The average deviation of individual determinations from the mean of duplicate determinations was 7.6 percent excepting the seed samples, with their relatively low selenium content, where this deviation was doubled.

Sulphur was determined by the method recommended by the Association of Official Agricultural Chemists for plant material. The average deviation of the determinations from the mean of duplicate determinations was 0.8 percent of the mean.

RESULTS

The selenium and sulphur determinations, expressed as percentages of the air-dry material, are recorded in tables 1 and 2. Table 1 contains the results of analyses of the tops of young plants cut at the ages of 1 and 2 months, while table 2 contains those obtained at 3 months, when some of the crops had developed to the point where it was desirable to analyze the leaves and the stems separately.

In both tables the selenium values for the different crops vary widely and, in general, parallel the correspondingly wide variation in the sulphur values. The latter differ in accordance with the great differences in sulphur absorption known to characterize the different plant groups (*3*, *7*, *8*). Thus the five members of the Cruciferae—cabbage, cauliflower, mustard, rape, and kale—have the highest percentages of both sulphur and selenium. Flax and sunflower are intermediate, as are the legumes except soybean. The last-named crop is of special interest because it took in so little sulphur as to fall in the class of the cereals instead of the legumes. Its selenium content was correspondingly low.

TABLE 1.—*Sulphur and selenium (based on air-dry weights) in the tops of crop plants 1 and 2 months old grown with 4 p. p. m. of selenium added as sodium selenate to Keyport clay loam*

Crop no. and name	1 month old		2 months old	
	Sulphur	Selenium	Sulphur	Selenium
	Percent	*Percent*	*Percent*	*Percent*
1, Cabbage (*Brassica oleracea capitata*)	3.37	0.0793	2.99	*0.0520
2, Cauliflower (*B. oleracea botrytis*)	2.88	*.0710	2.30	.0470
3, Black mustard (*B. nigra*)	2.78	.0744	1.97	.0435
4, Rape (*B. napus*)	2.39	.0780	1.86	.0585
5, Kale (*B. oleracea acephla*)	2.24	.0615	2.49	.0465
6, Flax (*Linum usitatissimum*)	1.28	.0474	1.32	.0358
7, Sunflower (*Helianthus annuus*)	1.32	.0310	1.18	.0275
8, Red clover (*Trifolium pratense*)	1.21	.0315		
9, Vetch (*Vicia villosa*)	.92	*.0280	.77	*.0150
10, Lettuce (*Lactuca sativa*)	.99	.0195	.73	*.0160
11, Wheat (*Triticum aestivum*)	.87	.0240	.84	.0225
12, Rye (*Secale cereale*)	.80	.0280	.60	.0195
13, Soybean (*Soja max*)	.59	.0130	.51	*.0140
14, Corn (*Zea mays*)	.42	.0140	.42	.0075
15, Sorgo (*Sorghum vulgare*)	.48	*.0130	.41	*.0070

* Single determinations.

TABLE 2.—*Sulphur and selenium (based on air-dry weights) in the tops of 3-month-old crop plants grown with 4 p. p. m. of selenium added as sodium selenate to Keyport clay loam*

Crop no. and name	Entire tops		Leaves		Stems		Seed	
	Sulphur	Selenium	Sulphur	Selenium	Sulphur	Selenium	Sulphur	Selenium
1, Cabbage (*Brassica oleracea capitata*)	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
	.....	.....	3.83	[b]0.0758	0.95	[b]0.0198	.....	.....
2, Kale (*B. oleracea acephla*)	.....	.....	3.72	[b].0720	.97	[a].0220	.....	.....
3, Rape (*B. napus*)	.....	.....	3.41	[b].0715	1.15	[a].0250	.....	.....
4, Cauliflower (*B. oleracea botrytis*)	.....	.....	3.38	[a].0630	.84	[a].0220	.....	.....
5, Black mustard (*B. nigra*)	.....	.....	2.64	[a].0480	1.24	[b].0223	1.38	0.0315
6, Sunflower (*Helianthus annuus*)	.....	.....	1.97	[b].0422	.75	[a].0100	.21	.0095
7, Onion (*Allium cepa*)	1.62	[a]0.0420	.....	.....	.....	.....	.....	.....
8, Flax (*Linum usitatissimum*)	1.23	[b].0245	.....	.....	.....	.....	.15	.0110
9, Alfalfa (*Medicago sativa*)	1.09	[b].0220	.....	.....	.....	.....	.....	.....
10, Red clover (*Trifolium pratense*)	.95	[a].0230	.....	.....	.....	.....	.....	.....
11, Vetch (*Vicia villosa*)	.89	.0245	.....	.....	.....	.....	.....	.....
12, Wheat (*Triticum aestivum*)	.78	[a].0120	.....	.....	.....	.....	.17	.0070
13, Rye (*Secale cereale*)	.64	[a].0110	.....	.....	.....	.....	.19	.0025
14, Millet (*Setaria italica*)	.62	[a].0150	.....	.....	.....	.....	.19	.0110
15, Lettuce (*Lactuca sativa*)	.61	[b].0093	.....	.....	.....	.....	.....	.....
16, Kafir (*Sorghum vulgare*)	.54	[a].0110	.....	.....	.....	.....	.15	.0025
17, Soybean (*Soja max*)	.46	[a].0110	.....	.....	.....	.....	.....	.....
18, Corn (*Zea mays*)	.....	.....	.63	[b].0160	.30	.0050	.13	.0035
19, Sorgo (*Sorghum vulgare*)	.....	.....	.36	[a].0110	.29	.0080	.12	.0050

[a] Single determinations.

[b] One of the duplicates was determined by a method (Greathouse) which generally gave higher values than did the method described in the present paper, especially for the high selenium concentrations. Therefore, those values that do not include such duplicates, notably those for mustard and cauliflower leaves, may be somewhat too low with respect to the rest.

When the selenium and sulphur percentages are plotted against each other in scatter diagrams, the tendency of the points to cluster about straight lines constitutes graphic evidence of a functional [6] relationship between the two elements (figs. 1 to 3). The average relationship between the variables was fairly constant for the plants of all three cuttings.

There was more of both sulphur and selenium in the leaves than in the stems, with least in the seeds of all the plants but mustard (table 2). The proportion of sulphur to selenium averaged practically the same for both leaves and stems, suggesting that the distribution of selenium in the plant coincides with the distribution of the sulphur-containing compounds.

TABLE 3.—*Relation between the amount of selenium available to plants (added to soil as sodium selenate) and the amount of selenium with reference to sulphur in their leaves*

[Soil sulphur=0.06 percent]

Soil treatment and crop	Sulphur in leaves	Selenium in leaves	Ratio of sulphur to selenium
With 3 p. p. m. selenium:	*Percent*	*Percent*	
1, Cabbage (young)	3.50	0.034	103
2, Broccoli	2.68	.022	122
3, Cabbage (old)	2.22	.018	123
4, Turnip	1.95	.024	81
5, Pea	.91	.006	152
6, Corn	.61	.006	102
7, Spinach	.63	.005	126
8, Lettuce	.51	.005	102
With 5 p. p. m. selenium:			
1, Broccoli	3.23	.133	24
2, Oats	1.48	.074	20
3, Barley	1.36	.064	21
4, Wheat	1.22	.055	22
5, Spinach	.90	.043	21

The absolute amount of selenium entering the plant varies with that in the soil, so that, with no change in the soil sulphur, the proportion

[6] This word is used in a mathematical and not a physiological sense.

of sulphur to selenium in the plant decreases with an increase of selenium in the soil. This proportion averaged well over 100 in soil treated with sodium selenate at the rate of 3 p. p. m. of selenium but only about 20 with 5 p. p. m. (table 3). Thus the value for one element can be fairly closely predicted from that of the other, once the interrelationship is established for the particular soil.

The consistency with which the proportion of selenium to sulphur in the plants varied with the concentration of selenium added to the

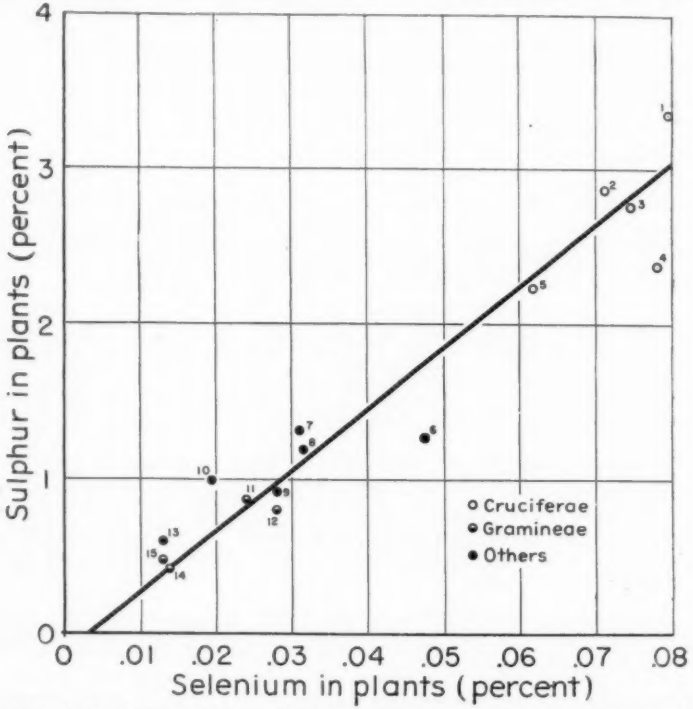

FIGURE 1.—Scatter diagram of selenium and sulphur percentages in the tops of 1-month-old crop plants listed in table 1, with line showing average relationship. (Numbers correspond to crop numbers in table 1.)

soil is illustrated by the scatter diagram in figure 4, which includes the data of table 3 for plants grown with 3 and 5 p. p. m. of selenium, and, for comparison, those of table 1 of similar age (2 months) grown with 4 p. p. m. The slope of the line of average relationship decreases progressively with 3, 4, and 5 p. p. m., respectively, of selenium in the soil.

It would perhaps be extending the implication of the data too far to conclude that the sulphur requirement determines the amount of selenium absorbed by all plants, including perennial species not represented in the present investigation. A plant of special interest in this connection is *Astragalus bisulcatus* A. Gray, a native perennial range plant of some of the seleniferous soils of the West, said to be capable of absorbing relatively enormous amounts of selenium under field conditions (*1*, *2*, *5*). One sample of such field material has been analyzed

for sulphur and found to contain an amount, 1.05 percent, that seems disproportionately low for its selenium content of 0.43 percent. But the soil producing these plants being unknown, some seed [7] was obtained from Wyoming and grown under the greenhouse conditions of the present experiments. The plants were analyzed after 7 months' growth and found to contain but 0.045 percent of selenium and 1.13 percent of sulphur—a proportion of sulphur to selenium sufficiently

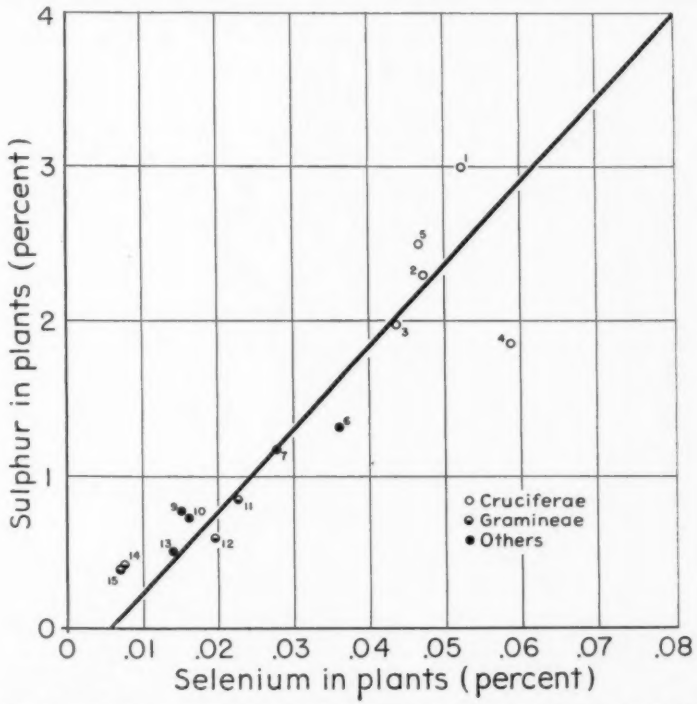

FIGURE 2.—Scatter diagram of selenium and sulphur percentages in the tops of 2-month-old crop plants listed in table 1, with line showing average relationship. (Numbers correspond to crop numbers in table 1.)

like that of the crop plants to indicate that *A. bisulcatus* grown in the greenhouse in artificially selenized soil did not constitute an exception to the generalization established by the crop plants.

DISCUSSION

The widely varying capacity of different types of crop plants to absorb sulphur is associated with a concomitant variation in capacity to absorb selenium. The sulphur found in the leaves of the 19 crops grown on Keyport clay loam untreated with respect to sulphur ranged from about 0.5 percent of the dry weight in certain of the Gramineae to over 3 percent in some of the Cruciferae. The quantities of selenium

[7] The seed was obtained through the courtesy of C. A. Kutzleb of the U. S. Forest Service. Its identity was corroborated by A. F. Musil of the Division of Seed Investigations on the basis of distinguishing characteristics of the seeds and seed pods.

taken up by these same plants (from 4 p. p. m. in the soil as sodium selenate) varied from about 0.01 percent in the low-sulphur Gramineae to about 0.07 percent in the Cruciferae, the figures tending to parallel those for sulphur throughout the range. This parallel variation in the rates of absorption of the two elements indicates a direct functional [8] relationship, explainable on the assumption that the plant does not discriminate between them in the processes of absorption and translocation. Since selenium and sulphur are adjacent elements in

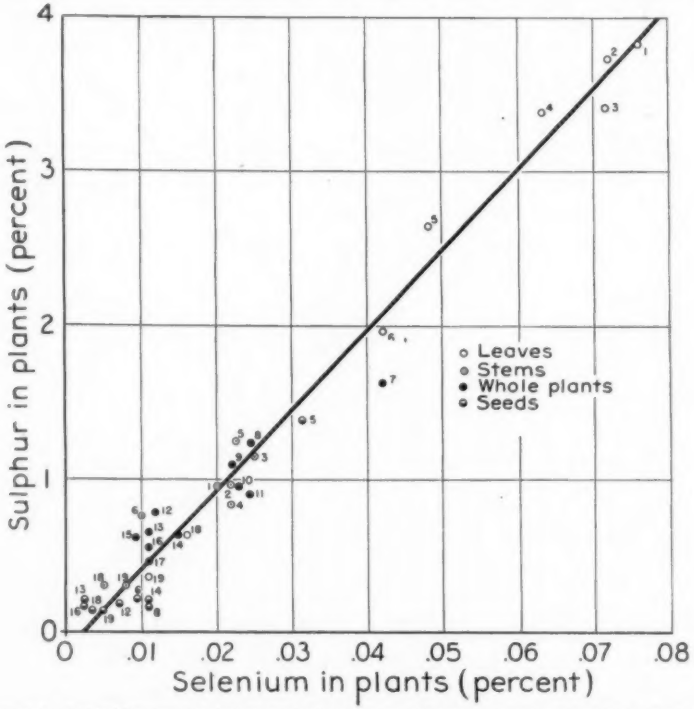

FIGURE 3.—Scatter diagram of selenium and sulphur percentages in leaves, stems, and seeds of various 3-month-old crop plants listed in table 2, with line showing average relationship. (Numbers correspond to crop numbers in table 2.)

the periodic table, it seems not unreasonable to assume that because of their chemical similarity the selenium and sulphur of the substratum enter alike in response to the sulphur gradient established by the plant's metabolism. (This hypothesis was proposed previously (*9*, *10*) in explanation of the phenomenon of sulphur-selenium antagonism.)

Since the possibility that the different crops might absorb other elements in this same order has not been eliminated, a causal connection between selenium and sulphur intake should not be considered proved by their parallel absorption rates. If, however, these rates are specific for the two elements the data may be interpreted as showing that the high selenium intake of the Cruciferae results from their large demand for sulphur, in accordance with the marked tendency of the

[8] See footnote 6.

group to form sulphur-containing compounds; and the lower selenium intake of such plants as the Gramineae is a consequence of their smaller demand for sulphur.

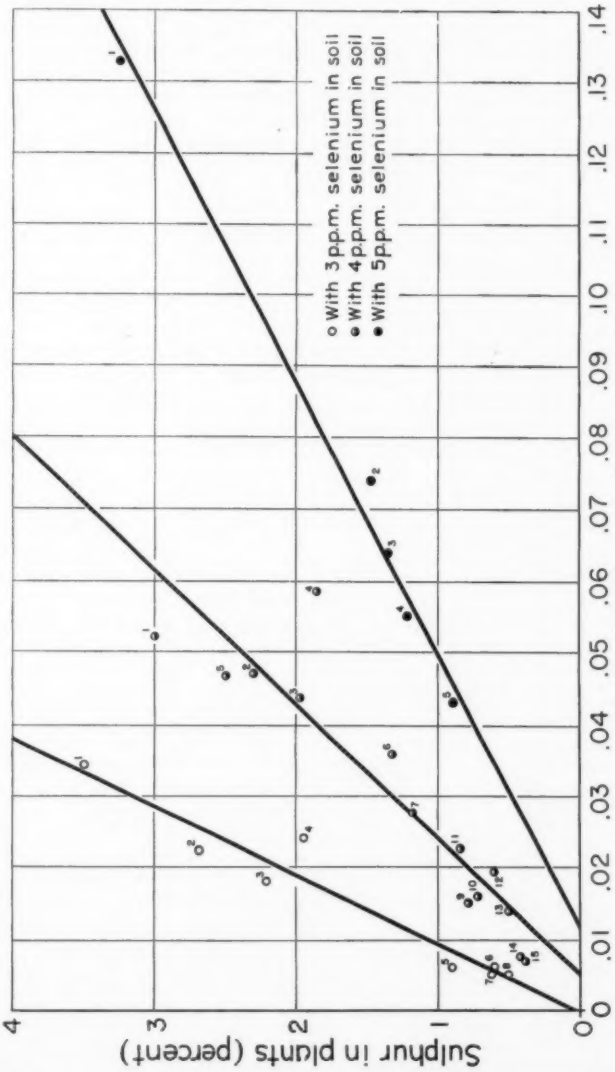

FIGURE 4.—Scatter diagram showing the relation between sulphur and selenium in plants grown with different concentrations of selenium as sodium selenate in the soil. (Numbers for the groups with 3 and 5 p. p. m. of selenium correspond to the crop numbers of table 3; those for the group with 4 p. p. m. are the crop numbers of table 1.)

From a practical standpoint, this relation permits prediction of the relative selenium absorption by crops whose sulphur requirements are known. Thus the growing of Cruciferae as food or forage crops in soils containing selenium would seem especially undesirable, since

toxicity of the food varies with its selenium content (*6*, *11*, *12*). The absolute amounts of selenium absorbed by the plants vary, of course, with the amount in the soil, as well as with the proportionate availability of sulphur.

SUMMARY

Wide differences in the absorption of selenium from sodium selenate by 19 different crop plants grown in Keyport clay loam, untreated with respect to sulphur, were directly correlated with corresponding differences in their respective sulphur-absorbing capacities. The parallelism suggests that the sulphur requirement of the plant determines its tendency to absorb selenium.

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DIASTATIC ACTIVITY OF ORANGE LEAVES AS AFFECTED BY TIME, TEMPERATURE, pH, AND CERTAIN ZINC SALTS [1]

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INTRODUCTION

Chemical analyses are sometimes incomplete indicators of biological reactions. In many cases enzyme activity affords a better insight into the mechanism of a biological reaction.

The present paper gives the results of experiments on the diastase of orange leaves, performed in order to determine whether diastatic analysis can be employed as a means of measuring the effect of treating citrus trees with various chemical agents, such as $ZnCl_2$ and $ZnSO_4$. Because zinc salts tend to precipitate proteins (*7, p. 101*),[2] including diastatic enzymes, and because of the physiological effect produced when citrus leaves which have been in a mottled condition (*8, 9*) are sprayed with $ZnSO_4$, it has been thought essential to obtain information on the diastase reaction of citrus leaves.

REVIEW OF LITERATURE

The chemical and physical properties of diastase from various sources, especially from the endosperm of seeds, have been investigated (*3, 11*), but information is lacking on factors affecting the diastatic activity of leaf tissues of such evergreens as *Citrus*. Harter (*6*) noted that in many deciduous plants the starch increased with the maturity of the leaf and with the approach of autumn; he reports that such a condition would necessitate a decrease in diastatic activity caused by the inhibitory action of oxidizing enzymes or by the accumulation in old leaves of products detrimental to diastatic activity. Cameron (*2*) has shown that a yearly variation of 0.66 to 2.96 percent may occur in the starch content of the leaves of young orange trees.

Bartholomew [3] has observed that when citrus plants are placed in the dark the leaves do not lose all of their starch overnight or within 2 or 3 days, as is the case with many plants. These plants contained a comparatively large amount of starch after they had been in continuous darkness for as long as 5 or 6 weeks. At the end of this time, when they began to turn yellow and fall, the leaves still contained much starch.

Experiments (*13, p. 103*) have been recorded which show that no consistent differences occur in the diastatic action of leaves during the 24-hour period and that no apparent correlation exists between the diastatic activity and the amount of starch and sugar in the leaves.

[1] Received for publication July 27, 1936; issued May, 1937. Paper no. 349. University of California Citrus Experiment Station and Graduate School of Tropical Agriculture, Riverside, Calif.

[2] Reference is made by number (italic) to Literature Cited, p. 618.

[3] BARTHOLOMEW, E. T. A STUDY OF THE ANATOMY OF CITRUS TREES WITH REFERENCE TO PATHOLOGICAL AND PHYSIOLOGICAL PROBLEMS. Unpublished data, Citrus Expt. Sta. Project 829. 1934.

However, young leaves may be more active diastatically than old ones, and fallen or dead leaves probably contain no diastase.

Denny (*4*) has observed that in certain annuals the total carbohydrate (sugar and polysaccharide) and dry weight of the leaves were nearly constant throughout the period preceding frost (autumnal migration). With the exception of a few cases, the nitrogenous substances showed losses of doubtful significance.

METHODS

Mature citrus leaves picked from eight different trees were minced as fine as possible by grinding them several times in a Wiley mill. Weighed samples to serve as controls were immediately placed in boiling 95-percent ethyl alcohol. Other samples of known amounts, within the range of 8 to 15 g, were taken from the same lot, and 100 cc of distilled water, previously warmed to 40° C., was added to each. The samples were then allowed to autolyze for different known periods of time up to 72 hours. At the end of the autolyzing period, the samples were poured into enough hot ethyl alcohol to produce an 85-percent solution (which was sufficient to allow the samples to stand until reducing substances could be determined).

Subsequently the alcoholic extract was evaporated to near dryness in vacuo at 55° C. The residue was taken up with hot distilled water, and the material adhering to the distillation flask was freed by shaking washed sand in the flask.

The aqueous extract was treated with lead acetate, and after this mixture had stood for an hour the precipitate was filtered and washed with warm water. The filtrate was then treated with Na_2HPO_4 to remove the excess lead. The precipitate thus formed was allowed to settle and was filtered and washed. The filtrate and washings were then diluted to a known volume, and aliquots were taken for copper determinations as prescribed by the method of Shaffer and Hartmann (*12*), with certain adaptations.

The reducing substances are expressed in terms of milligrams of copper. The copper was determined by extracting the control and autolyzed samples with 80-percent alcohol, as prescribed for sugar determinations. The nature of this material necessitated the making of a change in the strength of $Na_2S_2O_3$. Instead of 0.1 normal $Na_2S_2O_3$, a strength of 0.05 or less was used. The sucrose in the samples was inverted with hydrochloric acid at room temperature, as prescribed by the official methods (*1*).

In the autolysis experiments, where the hydrogen-ion concentration was regulated, 50 cc of acetic acid-sodium hydroxide buffer solution at pH 4.6 to 4.7 and 50 cc of distilled water were added to the leaf tissue. In the experiments containing taka diastase, 50 cc of the buffer solution and 50 cc of water containing 0.1 g of taka diastase were used; and in the experiments with soluble starch, 50 cc of 0.25-percent soluble starch solution and 50 cc of the buffer solution with pH 4.7 were added to the leaf tissue.

As commercial taka diastase was found to contain lactose as a diluent, which made it unsatisfactory for the conversion of starch in the tissue, it was discarded in favor of a sample of taka diastase[4] with an exceedingly high activity (815 percent) and a very low copper-reducing value.

[4] The sample was kindly supplied by Parke, Davis & Co. research laboratories.

EXPERIMENTAL

OPTIMUM pH

The diastase of orange leaves was active at pH 3 to 8. However, the reducing substances produced at pH 3 and 8, respectively, were comparatively slight, the greatest activity occurring at pH 4.0 to 5.2. In their earlier experiments the authors found no significant difference in the diastatic activity of the leaf within this range. In the experiments reported in this paper, in which the hydrogen-ion concentration was regulated, the solutions were adjusted to pH 4.7.

TIME OF REACTION

In order to obtain comparable results, the time of reaction is of considerable importance. Figure 1 shows that on incubation and

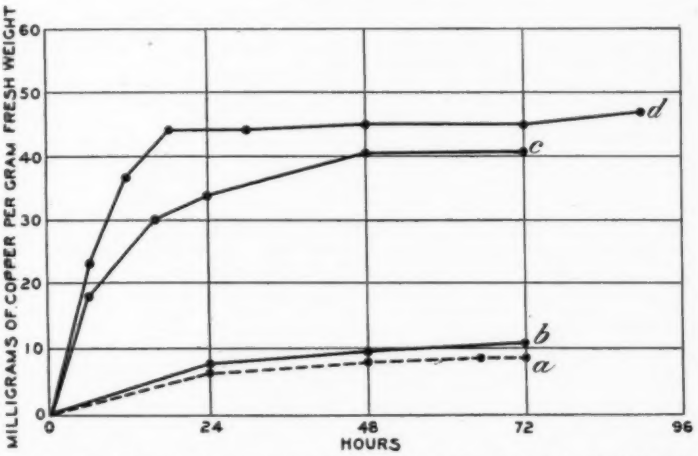

FIGURE 1.—*a*, Autolysis of leaf tissue in 100 cc of distilled water at 40° C.; *b*, autolysis of leaf tissue at pH 4.7 and at 40°; *c*, leaf tissue incubated at 40° and pH 4.7, with 50 cc of 0.25-percent soluble starch solution *d*, leaf tissue incubated at 40° C. and pH 4.7, with 0.1 g. of taka diastase in a 50-cc volume.

autolysis of leaf tissue the greatest activity occurred within 24 hours, although about 48 hours was required for the reaction to reach equilibrium. Only a slight amount of reducing substances was produced during the period from 48 to 90 hours.

INCUBATION AND AUTOLYSIS OF LEAF TISSUE

The autolysis of macerated leaf tissue under various experimental conditions is recorded in table 1. Series A shows the amount of copper recovered from orange-leaf tissue autolyzed for various lengths of time in 100 cc of distilled water. The hydrogen-ion concentration of these solutions ranged from pH 5.8 to 6.0. Series B records the milligrams of copper recovered when the leaf tissue was autolyzed in 50 cc of distilled water and 50 cc of acetic–sodium acetate buffer at pH 4.7 and a temperature of 40° C. Series C shows the results of autolyzing the leaf tissue in 50 cc of acetic–sodium acetate buffer at

pH 4.7 and 50 cc of 0.25 percent soluble starch solution. The series D experiments are the same as those in series C except that 0.1 g of taka diastase per 50 cc of volume was used instead of the starch solution.

TABLE 1.—*Reducing substances from Valencia orange leaves before and after autolysis*

Experiment no.	Time of autolysis	Copper per gram of fresh weight—		
		Before autolysis	After autolysis	Difference
	Hours	*Milligrams*	*Milligrams*	*Milligrams*
SERIES A, AUTOLYSIS IN 100 CC OF DISTILLED WATER AT 40° C				
1	24	43.40	49.71	6.31
2	48	43.80	51.75	7.95
3	72	44.12	52.60	8.48
SERIES B, AUTOLYSIS AT pH 4.7 AND 40° C				
1	24	42.77	49.91	7.14
2	48	42.31	52.24	9.93
3	72	42.85	53.20	10.35
SERIES C, AUTOLYSIS AT 40° C. AND pH 4.7 WITH 50 CC OF 0.25 PERCENT SOLUBLE STARCH SOLUTION				
1	6	50.86	68.64	17.78
2	17	50.86	80.56	29.70
3	24	80.02	113.73	33.71
4	48	80.42	120.49	40.07
5	72	77.43	118.32	40.89
SERIES D, AUTOLYSIS AT 40° C. AND pH 4.7 WITH 0.1 G OF TAKA DIASTASE IN A 50 CC VOLUME				
1	6	52.50	75.21	22.71
2	12	43.50	79.83	36.33
3	18	43.74	87.34	43.60
4	30	44.38	87.79	43.41
5	48	44.57	89.28	44.71
6	72	44.38	89.64	45.26
7	90	44.57	91.16	46.59

Figure 1 represents the data recorded in table 1 graphically by showing milligrams of copper per gram of fresh weight recovered on autolysis of leaf tissue plotted against the time of autolysis at 40° C.

TEMPERATURE

Table 2 shows the effect of varying the temperature from 3° to 70° C. upon the diastatic activity of citrus leaves. The reducing substances were obtained by allowing macerated Valencia orange leaves to act on 50 cc of 0.25-percent soluble starch at pH 4.7 for a period of 24 hours. Blank determinations were made on each sample of leaf tissue before incubation, and all figures reported in the table are corrected to a moisture content of 60 percent. At the end of the

TABLE 2.—*Reducing substances from Valencia orange leaves before and after autolysis for 24 hours* [1]

Experiment no.	Temperature of autolysis	Copper per gram of fresh weight		
		Before autolysis	After autolysis	Difference
	° C.	*Milligrams*	*Milligrams*	*Milligrams*
1	3	68.32	97.02	28.70
2	10	68.99	102.72	33.73
3	20	69.75	109.25	39.50
4	30	68.32	113.44	45.12
5	40	58.39	109.15	50.76
6	45	58.39	113.70	55.31
7	55	47.34	118.20	70.86
8	60	69.95	161.06	91.11
9	63	45.60	145.20	99.60
10	70	45.19	80.09	34.90
11	75	46.55	73.85	27.30
SERIES B, MATURE LEAVES (10 TO 12 MONTHS OLD)				
11	30	69.95	106.21	36.26
12	40	47.34	88.96	41.62
13	45	49.05	92.67	43.62
14	55	45.63	106.82	61.19

[1] Leaf tissue autolyzed at pH 4.7 with 50 cc of 0.25-percent soluble starch solution.

incubation period, the action of the enzyme was destroyed by pouring the samples into 10 times their volume of boiling 95-percent alcohol. In the table the diastatic values for the mature leaves are reported up to 55°. Values determined at higher temperatures show the same type of curve as the young leaves. The results are represented graphically in figure 2.

EFFECT OF ZINC SALTS ON THE ACTIVITY OF TAKA DIASTASE

The activity of taka diastase on 0.25-percent starch solution at pH 4.7 in the presence of various amounts of $ZnCl_2$ and $ZnSO_4$ is shown in table 3. The first column in series A shows the concentration of $ZnCl_2$ calculated as milligram equivalents of zinc per liter. Series B shows the same conditions for $ZnSO_4$.

These experiments were performed by putting 25 cc of acetic acid–sodium acetate buffer solution at pH 4.7 into a 100-cc Erlenmeyer flask. To this were added 25 cc of 0.25-percent soluble starch solution, 50 mg of taka diastase (815 percent), and the requisite quantity of $ZnCl_2$ or $ZnSO_4$, and finally a little toluene to act as a preservative. The total volume of the solution was about 60 cc. The flasks were placed in a constant-temperature oven at 40° C. and were shaken regularly. At the end of the incubation period, the samples were poured into eight times their volume of boiling 95-percent ethyl alcohol to destroy the enzyme action; they were then saved for subsequent determination of the copper equivalent. The values reported here were obtained in the presence of $ZnCl_2$, and they were repeatedly verified by incubating the material for 24, 48, and 68 hours, respectively. All three series yielded the same amounts of

reducing substances, within experimental error. The figures for the $ZnSO_4$ solution represent values obtained after an incubation period of 48 hours.

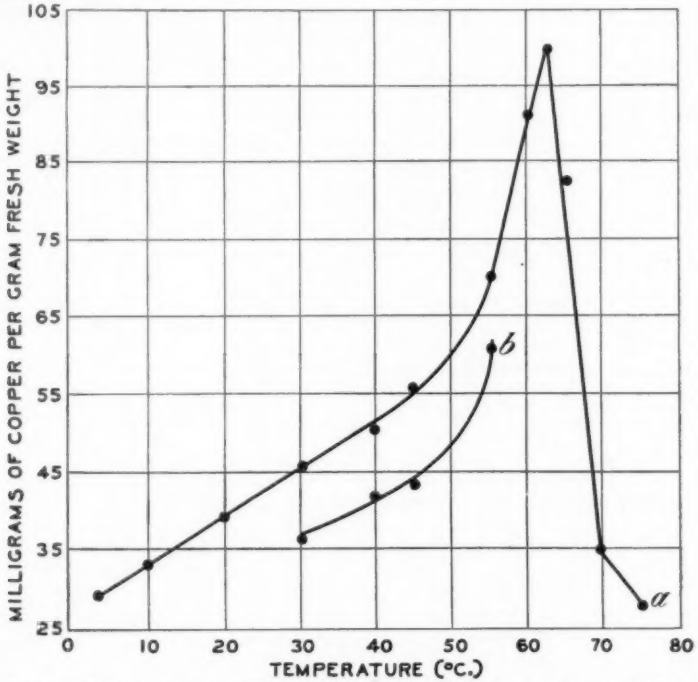

FIGURE 2.—The effect of temperature on the diastatic activity of (*a*) young and (*b*) mature Valencia orange leaves. The leaf tissue was incubated 24 hours at pH 4.7 with 50 cc of 0.25-percent soluble starch solution.

TABLE 3.—*The activity of taka diastase in the presence of various amounts of* $ZnCl_2$ *and* $ZnSO_4$

Zinc per liter (milligram equivalents)	Starch	Total reducing substance, copper	Glucose equivalent	Starch equivalent	Starch converted
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*	*Percent*
SERIES A, INCUBATED IN $ZnCl_2$ SOLUTION					
0.00	62.5	132.4	65.4	60.8	97.28
5.00	62.5	134.0	66.2	61.6	98.56
10.00	62.5	133.0	65.7	61.1	97.76
13.68	62.5	132.4	65.4	60.8	97.28
20.00	62.5	134.8	66.7	62.0	99.20
30.00	62.5	133.6	66.1	61.5	98.40
SERIES B, INCUBATED IN $ZnSO_4$ SOLUTION					
0.00	62.5	136.0	67.3	62.6	100.16
4.95	62.5	134.0	66.2	61.6	98.56
10.00	62.5	133.0	65.7	61.1	97.76
15.75	62.5	133.6	66.1	61.5	98.40
20.00	62.5	134.8	66.7	62.0	99.20
30.00	62.5	128.5	63.8	59.3	94.88

EFFECT OF ZINC SALTS ON THE ACTIVITY OF DIASTASE OF CITRUS LEAVES

Known weights of finely ground, fresh leaf tissue were placed in 300-cc Erlenmeyer flasks with 50 cc of sodium acetate buffer solution at pH 4.7 and 50 cc of 0.25-percent soluble starch solution. Separate determinations were made with the addition of various amounts of

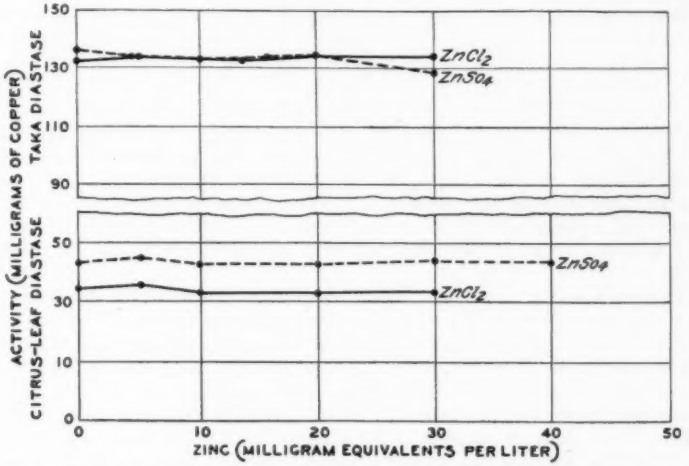

FIGURE 3.—Comparative activity of citrus-leaf diastase and taka diastase on soluble starch in the presence of various amounts of $ZnCl_2$ and $ZnSO_4$.

TABLE 4.—*The activity of citrus-leaf diastase on soluble starch in the presence of various amounts of $ZnCl_2$ and $ZnSO_4$ at pH 4.7 and 40° C.*

Experiment no.—	Zinc per liter	Copper		
		Before autolysis	After autolysis	Difference
	Milligram equivalents	*Milligrams*	*Milligrams*	*Milligrams*
SERIES A, INCUBATED IN $ZnCl_2$ SOLUTION AT 40° C.				
1	0	42.54	77.07	34.53
2	5	43.19	79.08	35.89
3	10	42.25	74.66	32.41
4	20	41.00	73.75	32.75
5	30	42.54	74.95	32.41
SERIES B, INCUBATED IN $ZnSO_4$ SOLUTION AT 40° C.				
1	0	53.22	96.65	43.43
2	10	53.22	98.67	45.45
3	20	51.00	94.65	43.65
4	30	52.55	96.76	44.21
5	40	52.55	96.04	43.49

$ZnCl_2$ and $ZnSO_4$. A few drops of toluene were added as a preservative. The flasks were incubated for 36 hours at 40° C. After the incubation period, the content of each flask was poured into eight times its volume of boiling 95-percent alcohol and reserved for the determination of the copper-reducing substances. The results of

these experiments, recorded in table 4, show the activity of citrus-leaf diastase on soluble starch in the presence of various concentrations of $ZnCl_2$ and $ZnSO_4$, expressed as milligram equivalents of zinc per liter. The values reported in table 4 are somewhat low, owing to the fact that these determinations were made in the fall of the year when the diastase was less active.

Some of the data in tables 3 and 4 are presented graphically in figure 3.

DISCUSSION

The curves in figure 1 were determined to find the best conditions for the diastatic activity of orange leaves. As shown in *a*, the autolysis of the macerated leaf tissue in distilled water hydrolyzes very little starch at the natural pH of the leaf tissue. This slight activity is partly due to inability to grind the wet tissue fine enough to enable rapid diffusion of the diastase to the starch particles, the latter, in all probability, being closely associated with the chloroplasts of the leaf cells.

Another factor worthy of consideration is that the starch exists in an insoluble form in the tissue. Investigators (*5*) have shown that starch should be gelatinized in order for the diastase to reduce the starch to simpler sugars. The heating of the tissue would, however, inactivate the enzyme. In the metabolism of the cell, conditions are such that the starch is readily acted upon by the diastase without rendering it soluble by heat. This is the case with wheat endosperm (*11*). The authors are well aware that in the quantitative determination of starch it is essential to gelatinize the material, but the experiments reported in this paper were concerned with the activity of the diastase and not with the absolute amount of starch in the orange leaves.

Curve *b* in figure 1 is the same as curve *a*, with the exception that the solution medium was regulated to pH 4.7. A slight increase in the amount of copper reduced was found, but not enough to account for the quantity of natural starch which this sample contained (3.70 percent, wet weight, as determined by the taka diastase method). Curves *a* and *b* represent reactions which are unsuitable for studying the diastatic activity.

In curve *c*, where the macerated tissue was incubated at pH 4.7 and 50 cc of 0.25-percent soluble starch solution were added to the medium, the diastase in the leaf cells readily diffused from the tissue and converted the starch to reducing substances. Curve *d* is similar to curve *c*, with the exception that to the former 0.1 g of taka diastase (815 percent) was added to the tissue instead of soluble starch. This added diastase was capable of actively converting the natural starch of the leaf to reducing substances. However, all of the starch in the leaf tissue was not acted upon by the diastase, for microscopic observation of iodine-treated residue showed that part of the starch in the interior of the leaf particles had remained unchanged. This confirms the idea that the problem of diffusion is of importance in relation to the conversion of the natural starch of the leaf by the natural diastase.

From the foregoing considerations it was decided that curve *c* represented the best conditions for the experimental study of the diastatic activity of orange leaves.

The relation of the diastatic activity to temperature is illustrated in figure 2. Rumsey (*11*) has recorded a similar curve on the rate of autolysis of wheat endosperm at pH 4.7, but the time of autolysis in his experiments was only 1 hour. Wheat flour (which is pure endosperm) has abundant starch and diastase, is finely ground, and is capable of producing an enormous diastatic activity within a short time. The experiments recorded in this paper and represented by figure 2 show that in young orange leaves the diastatic activity is proportional to the temperature up to about 55° C., the maximum activity occurring at about 63°. Curve *b* (fig. 2) is the type produced with mature leaves, which contain less diastase than young leaves.

The minimum time of incubation in the experiments was 24 hours instead of 1 hour, as reported by Rumsey (*11*) for endosperm of wheat. Since leaf tissue contains much less diastase than wheat endosperm, it was necessary to incubate the tissue not less than 24 hours in order to obtain an appreciable quantity of reducing substances. Figure 1 shows that most of the activity occurred within 24 hours.

Citrus-leaf diastase is active within a wide range of hydrogen-ion concentration. Comparable experimental results were obtained as long as the solutions were within the limits of pH 4.0 to 5.2. This is in accord with the observations of Rumsey (*11*), who showed that pH 4.7 was the optimum for the diastatic activity of wheat endosperm, and of Yemm (*14*), who employed pH 5.2 for determining the action of taka diastase on starch in plant residues.

Why citrus leaves should retain their starch so tenaciously, even when the plants are placed in darkness, is not known. Of course, as the leaves grow older the diastase may become less active, but the leaves tested were not over 1 year old and normally would have lived from 1 to 2 years longer. The long retention in the darkroom finally may have had a retarding effect upon the diastase in the leaves, but this does not explain why the starch did not disappear within the first 2 or 3 days. The inability of the diastase to contact the starch readily may be an important governing factor.

Many previous investigators (*15*) have reported the physiological significance of small amounts of the rarer elements in plant metabolism. Especially significant is the fact that when citrus trees are sprayed with solutions of certain salts, such as $ZnSO_4$, a marked stimulation in the biochemical reactions occurs. Mottled leaves of citrus show characteristic cytological changes (*10*) followed by an increase in the rate of growth. These facts indicate the importance of studying the effect of $ZnSO_4$ and $ZnCl_2$ on the enzyme reactions most concerned with the utilization of energy, such as diastase and catalase. So far experiments have been performed only on the starch-splitting enzyme, diastase, occurring in the leaves of citrus trees.

It was early found necessary and desirable to standardize the reaction with a highly active sample of taka diastase (815 percent). Up to 30 mg equivalents of zinc per liter in the form of $ZnSO_4$ and $ZnCl_2$, respectively, no paralyzing effect occurred in the activity of the taka diastase. Furthermore, this special taka diastase converted the starch quantitatively to glucose (table 3). These observations confirm the findings of Denny (*4*) and Yemm (*14*). Similar experiments were made with ground leaf tissue as a source of enzyme instead of taka diastase. The results recorded in table 4 show that there was

practically no difference in the amounts of copper reduced in the presence of 0 to 30 mg equivalents of zinc per gram of fresh weight of leaf tissue.

SUMMARY

Experiments have been performed to determine the optimum conditions, such as pH, temperature, and time, for the activity of citrus-leaf diastase. No significant difference occurred in the diastatic activity of citrus leaves within a range of pH 4.0 to 5.4. With macerated citrus-leaf tissue about 48 hours is required to reach equilibrium. The diastase of citrus leaves has a maximum activity between 60° and 65° C. The young leaves have a much greater diastatic activity than the mature leaves. Both kinds of leaves, however, show similar responses to temperature.

Determinations have shown that citrus leaves possessing adequate amounts of starch and diastase show little activity on autolysis of the macerated tissue in distilled water. The natural starch of the leaf appears to be bound by the chloroplast and is not reacted upon to any considerable extent by the naturally occurring diastase. A small increase in activity is observed when the hydrogen-ion concentration is regulated to pH 4.7, but if taka diastase is added to macerated leaf tissue at pH 4.7 the natural starch is hydrolyzed. However, if samples of macerated citrus-leaf tissue are added to a starch solution of pH 4.7 and allowed to incubate, the enzyme in the leaf converts the starch to reducing substances.

In concentrations of zinc containing as much as 30 mg equivalents of zinc per liter, taka diastase is capable of converting starch to glucose quantitatively. These concentrations of zinc appear to produce no paralyzing effect on the activity of the enzyme.

The diastase in macerated leaf tissue produced about the same quantities of reducing substances in concentrations of zinc containing from 0 to 30 mg equivalents per liter. These experiments were performed in $ZnCl_2$ and $ZnSO_4$ solutions.

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THE NITROGEN METABOLISM OF THE CROWN GALL AND HAIRY ROOT BACTERIA[1]

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INTRODUCTION

In studying so complex a phenomenon as pathological cell multiplication by crown gall and hairy root bacteria, it is desirable to learn everything possible about the metabolism of these organisms. Although many aspects of their metabolism are similar to those of nonpathogenic bacteria, these changes undoubtedly serve as a background for the pathological activities of the organism. This general question is reviewed by Riker and Berge (*13*).[2]

Information is already available about one or another of these bacteria in respect to pathogenicity (*12*), types of growth (*18*), titratable acidity and hydrogen-ion concentration induced in various media (*9, 11, 12, 14, 17*), changes in the oxidation-reduction potentials of several liquid media (*9, 11, 14*), types and amounts of carbon compounds fermented (*11, 12, 14*), and types of nitrogen compounds (*11, 14*) utilized. The crown gall organism utilizes various forms of inorganic nitrogen, but neither the crown gall nor the hairy-root bacteria liquefy gelatin or digest much, if any, casein; but under certain conditions all produce ammonia (*17*).

The work reported in the present paper is part of a larger program (*13*) on cell stimulation in plants. Its purpose is to present quantitative data regarding the utilization of various forms of nitrogen by these bacteria and to show the effect of glucose on the course of the nitrogen metabolism.

EXPERIMENTAL PROCEDURES

The bacteria employed in these studies were cultures of *Phytomonas tumefaciens* (Smith and Town). Bergey et al. and of *Phytomonas rhizogenes* R. B. W. K. and S. An attenuated sister crown gall culture was used parallel to the pathogenic culture. Various media were employed which had the following formulas:

Medium A.—

Calcium sulphate ($CaSO_4$)	0.1 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2 g.
Sodium chloride (NaCl)	0.2 g.
Sodium phosphate (NaH_2PO_4) 2.9 g in	13.0 cc
Potassium phosphate (K_2HPO_4) 8.8 g in	37.0 cc
20-percent yeast infusion	500.0 cc
Water	450.0 cc

The hydrogen-ion concentration was approximately pH 6.6. The 20-percent yeast infusion was the clear supernatant fluid resulting after 1,000 g of starch-free yeast had been autoclaved for several hours in 5,000 cc of water.

Medium B.—Like A except that 10 g of glucose was added.

Medium C.—Like A except that 1.1 g of ammonium nitrate was added.

[1] Received for publication July 31, 1936; issued May, 1937. This work has received support through grants from the International Cancer Research Foundation and from the special research fund of the University of Wisconsin.

[2] Reference is made by number (italic) to Literature Cited, p. 627.

Medium D.—Like A except that 1.1 g of ammonium nitrate and 10 g of glucose were added.

Medium E.—Like A except that 2.0 g of ammonium citrate was added.

Medium F.—Like A except that 2.0 g of ammonium citrate and 10 g of glucose were added.

Medium G.—Like A except that the yeast infusion was replaced with 500 cc of water, and 2.7 g of ammonium nitrate and 10 g of glucose were added.

Medium H.—Like A except that the yeast infusion was replaced with 500 cc of water, and 10 g of peptone was added.

The media, in 100-cc quantities in 250-cc flasks, were autoclaved for 1 hour at 15 pounds pressure. After seeding, they were incubated for 30 days at room temperature (about 20° C.).

The bacterial cultures studied were progenies of single cells. The hairy root and crown gall cultures C–1 and A–1, respectively, were those obtained by Wright et al. (*18*). The crown gall culture A–6–6, which has reduced pathogenicity or none when it is puncture-inoculated into tomato stems, was obtained by Hendrickson et al. (*9*). For the sake of brevity this culture is referred to as "attenuated." The characters of the cultures were checked at frequent intervals during the course of the investigation by inoculations of suitable host plants.

At the end of the incubation period for the various cultures in the media described the following determinations were made: Total nitrogen by the Folin and Farmer method (*6*); nonprotein nitrogen by the method of Folin and Wu (*8*); amino nitrogen by the Brown formol titration method (*4*); ammonia nitrogen by the Folin and MacCallum method (*7*); glucose by the method of Stiles, Peterson, and Fred (*16*); and pH by means of the quinhydrone electrode. In media containing nitrates the total and nonprotein nitrogen determinations were modified to include nitrates—acidification, evaporation, and addition of salicylic acid (*1*). All analyses were made in triplicate.

The determination of the above forms of nitrogen permits a distribution of the nitrogen of the culture medium into six groups (*10*), as follows: (a) Total nitrogen, a direct determination; (b) protein nitrogen (more accurately, nitrogen precipitable by tungstic acid but often designated as protein nitrogen), the difference between the total and nonprotein nitrogen (a minus c); (c) nonprotein nitrogen, a direct determination after removal of protein nitrogen; (d) difference nitrogen, the difference between the nonprotein nitrogen and the sum of the amino and ammonia nitrogen (c minus e and f). The largest constituent of the difference fraction is peptide nitrogen, but it also includes the nonamino nitrogen of amino acids and other undetermined nitrogen. In media to which ammonium nitrate was added, the nitrate nitrogen is included in this figure: (e) Amino nitrogen, a direct determination and subtraction of the ammonia nitrogen; (f) ammonia nitrogen, by direct determination.

CHANGES INDUCED IN VARIOUS MEDIA

CHANGES IN THE NITROGEN OF YEAST INFUSION

Since yeast infusion is one of the most satisfactory media for the growth of the crown gall organism, it was chosen as the basal medium in which to study the effect of glucose and ammonium salts on the course of the nitrogen metabolism. Table 1 shows the changes induced in 10 percent yeast infusion to which nothing had been added except the inorganic salts listed earlier.

TABLE 1.—*Changes induced by crown gall and hairy root organisms in the nitrogen of yeast infusion, of certain ammonium salts, and of peptone in the various media* [1]

YEAST INFUSION (MEDIUM A)

Micro-organism	pH	Total nitrogen	Protein nitrogen	Non-protein nitrogen	Difference nitrogen	Amino nitrogen	Ammonia nitrogen	Glucose unfermented
		Mg	*Mg*	*Mg*	*Mg*	*Mg*	*Mg*	*Mg*
None	6.8	128.2	5.2	123.0	99.2	15.6	8.2	----------
Crown gall	7.7	128.2	39.8	88.4	46.2	.4	41.8	----------
Attenuated crown gall	7.7	128.2	39.8	88.4	44.1	.6	43.7	----------
Hairy root	7.6	128.2	32.1	96.1	53.9	1.9	40.3	----------
YEAST INFUSION IN THE PRESENCE OF GLUCOSE (MEDIUM B)								
None	6.6	130.5	5.0	125.5	103.5	16.7	5.3	1,090.0
Crown gall	6.8	130.5	41.5	89.0	83.0	4.6	1.4	58.8
Attenuated crown gall	6.8	130.5	45.1	85.4	74.4	4.7	4.3	45.8
Hairy root	6.8	130.5	5.0	125.5	101.5	19.7	4.3	705.0
YEAST INFUSION AND AMMONIUM NITRATE (MEDIUM C)								
None	6.5	156.3	4.7	151.6	113.8	13.8	24.0	----------
Crown gall	6.9	156.3	44.1	112.2	68.5	1.5	42.2	----------
Attenuated crown gall	7.0	156.3	47.2	109.1	60.0	.0	49.1	----------
Hairy root	7.1	156.3	31.3	125.0	70.9	.4	53.7	----------
YEAST INFUSION AND AMMONIUM NITRATE IN THE PRESENCE OF GLUCOSE (MEDIUM D)								
None	6.6	159.0	4.8	154.2	112.4	17.2	24.6	985.0
Crown gall	6.5	159.0	46.3	112.7	96.3	2.1	14.3	63.7
Attenuated crown gall	6.5	159.0	47.1	111.9	94.3	4.0	13.7	59.2
Hairy root	6.4	159.0	25.8	133.2	99.4	22.9	10.9	437.0
YEAST INFUSION AND AMMONIUM CITRATE (MEDIUM E)								
None	6.7	143.1	4.1	139.0	89.2	11.5	38.3	----------
Crown gall	7.5	143.1	40.9	102.2	46.1	1.1	55.0	----------
Attenuated crown gall	7.5	143.1	42.2	100.9	44.3	1.0	55.5	----------
Hairy root	7.6	143.1	35.1	108.0	52.5	1.5	56.0	----------
YEAST INFUSION AND AMMONIUM CITRATE IN THE PRESENCE OF GLUCOSE (MEDIUM F)								
None	6.8	145.5	4.5	141.0	85.7	15.3	40.0	970.0
Crown gall	6.9	145.5	41.1	104.4	75.3	7.8	21.3	75.1
Attenuated crown gall	6.9	145.5	40.3	105.2	76.1	6.3	22.8	70.3
Hairy root	6.5	145.5	29.3	116.2	82.1	19.0	25.2	684.0
AMMONIUM NITRATE IN THE PRESENCE OF GLUCOSE (MEDIUM G)								
None	6.9	91.3	0	91.3	46.1	0	45.2	956.0
Crown gall	5.5	91.3	20.5	70.8	39.3	0	31.5	521.0
Attenuated crown gall	5.6	91.3	16.5	74.8	42.2	0	32.6	384.0
PEPTONE (MEDIUM H)								
None	6.6	144.0	64.7	79.3	59.8	16.1	3.4	----------
Crown gall	7.9	144.0	50.2	93.8	44.8	4.9	44.1	----------
Attenuated crown gall	7.9	144.0	51.5	92.5	43.7	5.1	43.7	----------
Hairy root	7.7	144.0	62.4	81.6	41.0	5.5	35.1	----------

[1] Values except pH in milligrams per 100 cc.

The most striking changes were the marked increases in ammonia and protein nitrogen. The increase in protein nitrogen represents in the main the nitrogen converted into cellular proteins while the ammonia nitrogen indicates in a general way the extent to which the amino acids and the polypeptides were utilized for energy. Amino nitrogen decreased almost to zero, a situation indicating that utilization of amino acids was more rapid than hydrolysis of protein derivatives. Difference nitrogen, which in this case consisted mainly of polypeptides, decreased to approximately half of its original value. Thus the polypeptides furnished most of the nitrogen utilized in the formation of bacterial cellular proteins and of ammonia.

EFFECT OF GLUCOSE ON NITROGEN METABOLISM IN YEAST INFUSION

In general, the addition of glucose to a medium containing proteins or their derivatives alters the course of the dissimilation. The results (table 1, medium B) show the sparing effect of glucose on the nitrogenous constituents. The ammonia content decreased, showing that if ammonia was produced, it was utilized for structural purposes. The protein nitrogen increased in the case of the crown gall and attenuated crown gall organisms to about the same degree as in the absence of glucose. The hairy root organism did not produce an abundant growth in this experiment; hence there was no increase in protein nitrogen, and instead of a decrease in the amino nitrogen, as was observed in the absence of glucose, a small increase occurred.

According to Berman and Rettger (*3*) and Slanetz and Rettger (*15*), proteins are spared by carbohydrates because acids produced from the latter inhibit the normal nitrogen metabolism. It is obvious from the data presented that this explanation does not apply here because neither the crown gall nor the attenuated crown gall organism produced acid. A more probable explanation is that glucose is preferred to protein as a source of energy. With glucose furnishing the main supply of energy for the organism, the breakdown of protein becomes unnecessary.

CHANGES IN THE NITROGEN OF YEAST INFUSION AND AMMONIUM NITRATE

It is to be expected that the addition of ammonium salts to a yeast-infusion medium would have little effect on the nitrogen metabolism, since the organisms under investigation produce ammonia. The results as shown in table 1 (medium C) are quite in accord with this expectation. There was an increase in ammonia and protein nitrogen and a decrease in nonprotein and amino nitrogen. Thus, the changes are analogous to those in yeast infusion.

EFFECT OF GLUCOSE AND AMMONIUM NITRATE ON NITROGEN METABOLISM IN YEAST INFUSION

When glucose was added to yeast infusion the ammonia content remained practically unchanged. Consequently, both glucose and ammonium salts were added to a yeast infusion medium to determine the effect of glucose on the utilization of ammonium salts.

The results (table 1, medium D) are similar in many respects to those recorded for medium B in table 1. In the presence of glucose about half of the added ammonium salt was used, while in the absence of glucose (table 1, medium C), additional ammonia was produced.

In previous work (*14*) it was found that the hairy root organism made little or no growth when ammonium salts were the sole source of nitrogen. The reason for this failure to grow does not lie in the availability of the ammonium salt, for in the presence of glucose and yeast infusion this organism used more of the ammonium salt than did the crown gall organism, although the latter will grow in a medium containing ammonium salts as the sole source of nitrogen.

CHANGES IN THE NITROGEN OF YEAST INFUSION AND AMMONIUM CITRATE

The addition of ammonium nitrate to media for the purpose of studying the effect of ammonium salts on the nitrogen metabolism is complicated by the fact that the nitrate nitrogen is available to both the crown gall and attenuated crown gall organisms and undoubtedly is utilized to some extent. Ammonium citrate does not involve this complication but nevertheless furnished a readily available source of nitrogen.

The inclusion of ammonium citrate in a yeast-infusion medium had comparatively little effect on the nitrogen distribution (table 1, medium E). Large amounts of ammonia and protein nitrogen were produced, with a corresponding decrease in "difference" and amino nitrogen. These changes are quite comparable to those which took place in plain yeast infusion, and in yeast infusion to which ammonium nitrate had been added (table 1, media A and C). The citrate radical had little if any sparing action on the protein fractions, and apparently is a less available source of carbon than is protein.

EFFECT OF GLUCOSE AND AMMONIUM CITRATE ON NITROGEN METABOLISM IN YEAST INFUSION

The addition of glucose to a medium containing yeast infusion and ammonium citrate should result in a sparing of the protein derivatives and a utilization of the ammonia similar to that found when glucose was added to a medium containing ammonium nitrate and yeast infusion. The results shown in table 1 (medium F) are in agreement with this view. While all of the organisms studied are capable of utilizing citric acid, it is evident that glucose was still the chief source of energy.

CHANGES IN THE NITROGEN OF AMMONIUM NITRATE

Both ammonium salts and nitrates are available to the crown gall and attenuated crown gall organisms in the presence of a suitable source of energy such as glucose. Since no information is available as to the amounts of either form of nitrogen used, nor the relative availability of the two forms of nitrogen, a study was made for the purpose of securing information on these points. The hairy root organism is unable to grow in a medium containing only inorganic nitrogen, hence no attempt was made to study its metabolism in this case.

Table 1 (medium G) shows the extent of utilization of ammonium salts when ammonium nitrate furnished the sole source of nitrogen. Since the growth on this medium was not so abundant as on media containing yeast infusion, only small amounts of protein were formed. The figures for glucose also indicate a reduced growth and fermentation.

The decrease in ammonia nitrogen is much greater than that of the difference nitrogen, which in this case consisted entirely of nitrate nitrogen, indicating that the organisms utilized ammonia nitrogen more readily than nitrate nitrogen. This conclusion is also borne out by the increase in hydrogen-ion concentration. Probably this increase was the result of the utilization of more ammonium ions than nitrate ions, which left an excess of hydrogen ions in solution. If small amounts of amino nitrogen were formed, they were not detected by the methods used.

CHANGES IN THE NITROGEN OF PEPTONE

The nitrogen metabolism of these organisms in a peptone medium (H) differed in only one respect from that in a yeast-infusion medium. In a yeast-infusion medium there was, in general, an increase in protein nitrogen, while in a peptone medium there was a small decrease. The cellular growth in this medium was much less abundant than in yeast infusion, and hydrolysis of "protein" was evidently greater than synthesis of cellular protein. Large decreases occurred in the amino and difference nitrogen and a corresponding increase in ammonia nitrogen. Such a correlation would appear inevitable as there was no source of energy other than the peptone.

DISCUSSION

The increase in protein nitrogen is a conspicuous feature of the nitrogen metabolism of these organisms. This increase consists probably of bacterial cells, autolysis products of the cells, and small amounts of soluble constitutents entrained in the tungstic acid precipitate.

No increase in protein nitrogen occurred in the peptone medium. Growth on this medium was somewhat less abundant than on yeast-infusion media, and if any hydrolysis of the larger protein derivatives occurred, there was an equivalent synthesis of cellular material.

The crown gall and its attenuated sister culture fermented large amounts of glucose in the media which contained yeast infusion. The hairy root organism did not ferment such large quantities of sugar; approximately 50 percent of the sugar remained unfermented. This lack of metabolic activity is also reflected in the figures for protein nitrogen. In the cases where a poor fermentation of glucose occurred, the protein nitrogen was likewise low.

While no safe conclusion can be drawn from the metabolism of an organism in culture concerning its metabolism in a host plant, it is not improbable that the metabolism in the host resembles that on the media containing glucose, ammonium salts, and yeast infusion more closely than that on any of the other media.

The similarity in the nitrogen metabolism of the crown gall and the attenuated culture is in accord with previous work (*9*) which indicated that the organisms were quite similar in cultural characteristics.

SUMMARY

Quantitative data have been obtained regarding the nitrogen metabolism of the crown gall, attenuated crown gall, and hairy root organisms, on eight different media.

In media containing glucose and yeast infusion, approximately one-fourth to one-third of the total nitrogen was converted into cellular

proteins. Amino nitrogen decreased in the case of the crown gall and attenuated crown gall organism. The hairy root organism did not cause a decrease in the amino nitrogen, indicating that the formation of amino nitrogen equaled its utilization. Ammonia nitrogen decreased slightly, and in media to which ammonium salts were added, utilization of the ammonia occurred.

In similar media but without glucose, large amounts of ammonia were formed. Protein nitrogen increased to about the same degree as in the presence of glucose.

The crown gall and attenuated crown gall organisms were able to utilize ammonium nitrate as the sole source of nitrogen in the presence of glucose. Ammonia nitrogen was more available than nitrate nitrogen.

The polypeptide and amino nitrogen of peptone was readily utilized by these organisms and resulted in an increase in cellular proteins and ammonia. The fraction precipitated by tungstic acid was less readily available.

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TWO MOSAIC DISEASES OF PEAS IN WASHINGTON[1]

By FOLKE JOHNSON, *research assistant*, and LEON K. JONES, *associate plant pathologist, Washington Agricultural Experiment Station*

INTRODUCTION

Investigations which have been in progress during the last 2 years in Washington have shown that two distinct mosaic diseases are prevalent on peas (*Pisum sativum* L.) in that State. Enation mosaic (*7*)[2] has so reduced the value of the crop in certain areas of western Washington in the last 3 years that from 10 to 50 percent of the pods have been classed as culls. Plants affected with the other type of mosaic, called in this paper "severe" mosaic, usually produce few if any salable pods. In 1935, pea fields in western Washington frequently showed from 5 to 15 percent of the plants affected with severe mosaic.

Peas grown for canning or for seed have shown much less injury from virus diseases than greenpod peas. Severe mosaic has been noted on 2 to 5 percent of the plants in many fields, and in 1935 a loss of about 50 percent of the crop was noted in one 40-acre field.

The few observations that have been made in eastern Washington on peas grown for the seed and split-pea industry have not shown the virus diseases to be very prevalent. In 1934, however, when aphid infestation was unusually heavy, severe mosaic was common in the plantings in the vicinity of Pullman.

SYMPTOMS

ENATION MOSAIC

Enation pea mosaic (*7*), formerly called common mosaic by the authors (*3*), appears to be the same as the pea mosaic discussed by Osborn (*6*), and similar to the one referred to as common pea mosaic (*12*). It differs, however, from the pea mosaic viruses described by Zaumeyer and Wade (*11, 12*) in that it systematically infects Corbett Refugee beans and causes enations to develop on affected pea plants.

The first symptoms appear on the youngest stipules and leaflets 10 to 13 days after inoculation as a fine mottle of light areas in the normal green tissue, and the leaflets remain folded for a longer period than leaflets on healthy plants. As the disease progresses the characteristic symptoms develop; i. e., small light-green areas scattered over the leaflets and stipules (fig. 1); puckering, curling, and rolling of terminal growth; and slight dwarfing of the plants (fig. 2, *A*). Irregular outgrowths of plant tissue (enations), as reported by Osborn (*6*), may appear on the under surface of leaves. The pods are malformed, with raised dark-green areas on the surfaces. Later they become curled and twisted in a way similar to that reported by Linford [3] and Snyder (*9*). The pods on severely affected plants fail

[1] Received for publication July 31, 1936; issued May, 1937. Published as Scientific Paper 347, College of Agriculture and Experiment Station, State College of Washington.

[2] Reference is made by number (italic) to Literature Cited, p. 637.

[3] LINFORD, M. B. PEA DISEASES IN THE UNITED STATES IN 1928. U. S. Dept. Agr., Bur. Plant Indus., Plant Disease Reptr. Sup. 67: 1-14. 1928.

to ripen uniformly, and have a reduced number of seeds, many of which shrivel and dry up.

SEVERE MOSAIC

Severe mosaic first appeared on plants grown in the greenhouse at the State College of Washington in Pullman in the fall of 1934, from seed collected in western Washington. The disease appears to be similar to that ascribed by Pierce (*7*) to pea virus 3 and by Zaumeyer and Wade (*12*) to white sweetclover mosaic. Mottling, dwarfing, and reduction in yield of pods are considerably more severe than with enation pea mosaic. Definite vein clearing of the youngest leaves is noticeable in 7 to 8 days after inoculation, and this is followed by a

FIGURE 1.—Symptoms of enation pea mosaic on Alderman 40 pea plants.

water-soaked appearance of the terminal growth, which later dries and gives a yellowed, tipburned appearance to the affected plant. Diseased plants often succumb, but they may continue to make a slow growth of severely mottled, somewhat dwarfed and curled foliage. Most of the leaf area develops a light-green to yellowish-green color, with a small amount of normal green adjacent to the veins (fig. 3). Usually diseased plants are severely dwarfed (fig. 2), often to such an extent that only a compact, rosetted growth of yellowish curled foliage is produced above the point of inoculation. Sometimes, however, a few leaves above the point of inoculation yellow and die and the plant grows through this area to produce a more normal appearing, yellowish, dwarfed plant.

SEED TRANSMISSION

Contradictory results have been found in the literature relative to seed transmission of pea mosaic, as follows: 3 to 76 percent transmission in tests with six strains of peas (*1*); 11 mosaic seedlings in tests

with 3,057 seeds (*11*); 0.8 percent and no transmission (*5*); no transmission in tests with six strains of peas (*2*).

During the season of 1933, 17 acres of Alderman 40 peas were grown in the vicinity of Pullman from seed produced in an area of western

FIGURE 2.—Effect of mosaic viruses on Perfection pea plants: *A*, Enation mosaic; *B*, check; *C*, severe mosaic.

Washington where mosaic was very abundant. Careful observations on this planting showed that 11 plants scattered over the 17 acres were affected with enation mosaic (fig. 1), which was probably associated with seed transmission.

Plantings to determine the importance of seed transmission of mosaic were made in the greenhouse at Pullman during the winters of 1934 and 1935 (table 1). A number of seedlings showed chlorotic

symptoms of various types, and in each case portions of the plants were used as inoculum on healthy plants. Only 1 of the 15,694 plants developed mosaic (severe mosaic) that could have originated from the seed.

To determine whether some of the seedlings might be symptomless carriers of the viruses a further experiment was made. One leaflet

FIGURE 3.—Severe mosaic of Perfection pea plant produced by inoculation with the virus after passage through Refugee Stringless Green Pod beans.

was removed from each of 500 of the plants grown from seed lot 9- These leaflets were frozen for 12 hours, and the cell sap was then ex. pressed at 2,000 pounds' pressure in a hydraulic press and used as inoculum on 50 healthy Morris Market pea plants, with a similar number of plants as controls. No mosaic developed on the plants during the 30 days in which they were observed.

In another experiment 100 seeds from lot 9 were soaked in water for 24 hours, frozen for 12 hours, and the cell sap extracted under pressure as described above. Inoculations were made with this extracted liquid to 50 Morris Market pea plants, but no mosaic symptoms developed.

TABLE 1.—*Extent of seed transmission of pea mosaic viruses in different pea varieties*

Variety	Lot	Year collected and tested	Source of seed	Plants	Mosiac plants
				Number	*Number*
Hundredfold	1	1934	Seedman's sample grown in area where mosaic was abundant.	397	0
Laxton Progress (sorted sample).	2	1934	do	267	1
Laxton Progress (culls)	3	1934	do	255	0
Alderman	4	1934	do	35	0
Stridah	5	1934	do	454	0
Dwarf Alderman	6	1934	Collected from plants known to have enation mosaic.	167	0
Tall Alderman	7	1934	Collected from fields in which nearly 100 percent of plants had enation mosaic.	3,986	0
Tall Telephone	8	1934	do	2,286	0
San Diego Bush	9	1935	Collected from plants showing 100 percent enation mosaic and 10 percent severe mosaic.	7,847	0

Seeds of Radio peas showing small, dark, water-soaked spots quite similar to the physiological spotting described by Zaumeyer and Wade (*10*), were obtained during the fall of 1934. Numerous culture tests from the discolored areas failed to show the presence of any pathogenic organism. The discolored areas were cut out of a few of these seeds, macerated in a mortar, and used as inoculum on healthy plants of the Stridah variety. These tests (table 2) showed a low percentage of transfer of the severe mosaic virus.

TABLE 2.—*Extent of seed transmission of pea mosaic viruses by inoculation in several pea varieties*

Variety of seed	Seeds used	Treatment of inoculum	Age of seed from pods	Plants inoculated	Mosaic plants	Check plants [1]
	Number		*Months*	*Number*	*Number*	*Number*
Radio	10	Spots cut out and macerated in mortar with pestle.	5	70	4	38
Do	50	Seed soaked in water, frozen, and juice extracted.	17	50	0	--------
Stridah	5	Pods inoculated with severe mosaic	[2]	51	21	16
San Diego Bush	100	Seed soaked in water, frozen, and juice extracted.	4	50	0	50
Do	50	Seed coats [3]	4	500	0	0
		Cotyledons [3]	4	500	0	0

[1] Check plants remained free of mosaic.
[2] Fresh.
[3] Seed coats removed aseptically following immersion in 40-percent formaldehyde. Each seed coat and each pair of cotyledons macerated separately and used as inoculum on 10 pea plants.

A further test was made by inoculating pods of Stridah peas with the severe mosaic virus while the pods were in the flat-pod stage. Each pod was rubbed with a sterile swab stick until the sides and midrib were slightly bruised, and the virus inoculum was then applied on a cotton swab in three places in the usual manner. Twenty-four days after inoculation the peas were removed from the pods, and a

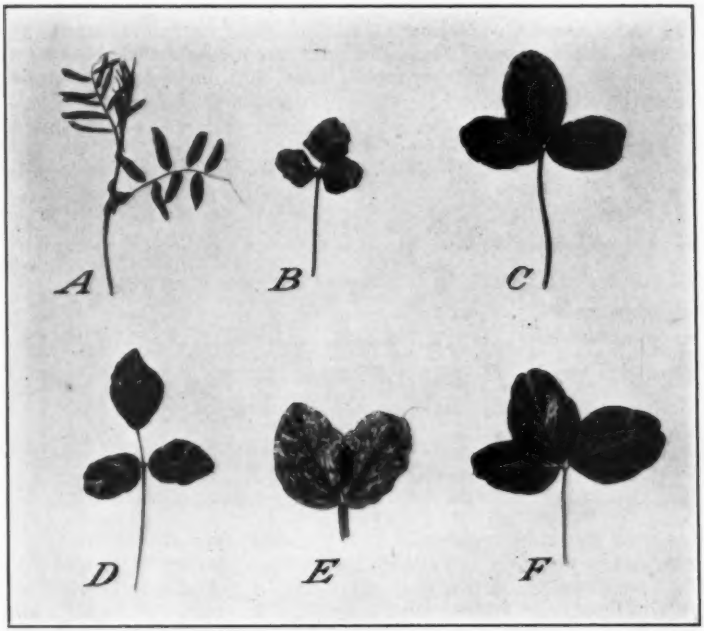

FIGURE 4.—Effect of severe mosaic virus upon other legumes: *A*, Vetch; *B*, hop clover; *C*, alsike clover *D*, sweetclover; *E*, sweet pea; *F*, red clover.

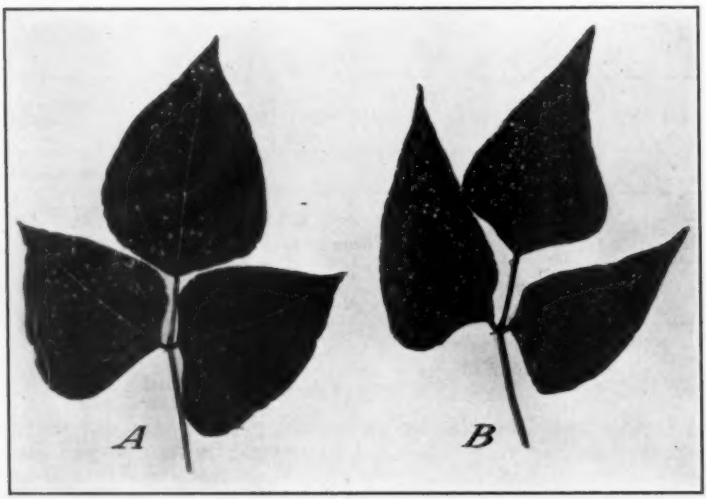

FIGURE 5.—Effect of severe mosaic virus upon foliage of Robust (*A*), and Refugee Stringless Green Pod (*B*), beans.

few of the seeds showed dark, water-soaked areas on the seed coat which resembled the spots noted on the Radio seed described above. Macerated portions of the seed from inoculated pods were used as inoculum on Stridah pea plants, and the results (table 2) show that the severe mosaic virus was present in the seed.

Tests with macerated seed, seed coats, and cotyledons from San Diego Bush seed collected from mosaic-infected plants in 1935 failed to show the presence of virus (table 2). The seed was collected from plants all of which were affected with enation mosaic, and 10 percent of which were also affected with severe mosaic.

It appears that mosaic viruses may be transmitted in pea seed, usually with low percentages of the seed affected, but that under most conditions seed transmission is probably not a factor in the spread of mosaic diseases.

HOST RANGE

Since seed transmission of the mosaic viruses does not account for the destructive development of these diseases, other sources must be sought. Probably other hosts, especially closely related leguminous plants, are very important in the overwintering and spread of the viruses to peas.

The results of inoculations of a number of leguminous plants are presented to show the partial host range of the two mosaic viruses as determined in these studies (table 3). The studies were all made under greenhouse conditions during the winter months with insect control by frequent fumigation with Nicofume and Cyanogas. All inoculations were made according to the method described by Jones (*4*) with the further adaptation of the use of carborundum dust as described by Rawlins and Tompkins (*8*). In each case of inoculation with the pea viruses to other hosts, portions of the inoculated plants, following a sufficient period of incubation, were used as inoculum back to peas as a final test of transmission.

TABLE 3.—*Results of inoculations with severe and enation mosaic viruses from pea on other varieties of plants*

Host [1]	Severe mosaic		Enation mosaic	
	Negative or positive [2]	Symptoms produced	Negative or positive [2]	Symptoms produced
Alfalfa (*Medicago sativa*)	+	Mottle, crinkle, dwarfing	+	Vein clearing.
Alsike clover (*Trifolium hybridum*)	+	do	+	Do.
Red clover (*T. pratense*)	+	Slight mottle, vein clearing	−	
White clover (*T. repens*)	+	Mottle, crinkle, dwarfing	−	
Yellow sweetclover (*Melilotus officinalis*)	+	do	−	
Yellow trefoil (*Medicago lupulina*)	+	do	−	
Vetch (*Vicia sativa*)	+	Mottle, dwarfing	−	
Lentil (*Lens esculenta*)	+	Mottle	−	
Horsebean (*Vicia faba*)	+	Mottle, dwarfing, necrosis	−	
Sweet pea (*Lathyrus odoratus*)	+	Mottle, dwarfing	+	Mild mottle.
Perennial pea (*Lathyrus latifolius*)	+	do	+	Do.
Chickpea (*Cicer arietinum*)	−		−	
Bean (*Phaseolus vulgaris*):				
Robust	+	Mottle, dwarfing	−	
Corbett Refugee	+	Small light area mottle	+	Mild mottle.
Refugee Stringless Green Pod	+	do	−	
Great Northern No. 1	+	Large green area mottle	−	
Tobacco (*Nicotiana tabacum*)	−		−	

[1] 12 to 20 plants of each host were inoculated with each pea virus.

[2] Inoculations were made back to peas (20 pea plants in each test) from each host series of plants that had been inoculated with the pea viruses.

The results of these cross inoculations show that the severe mosaic virus has a very wide host range (table 3 and figs. 4 and 5), whereas only a few of the legumes tested were susceptible to the enation mosaic virus.

CHARACTERS OF THE VIRUSES

In the study of the characters of the viruses a uniform procedure was used in extracting plant juices and in inoculation tests. During the fall of 1934 the selected samples of tissue from affected and control plants was frozen in the refrigerator for 12 hours and the juice extracted under 2,000 pounds' pressure in a hydraulic press. Negative results from inoculations with the enation virus showed that possibly this virus was inactivated during this period of treatment. Later tests have been made with juice extracted in the hydraulic press without freezing. All inoculations were made by the sterile-swab and pot-label method (*4*) following the addition of a small amount of carborundum dust to the foliage to be inoculated.

TABLE 4.—*Longevity of pea mosaic viruses in vitro and in dry plant tissue*

Virus tested	Positive or negative results after indicated period of aging [1]							
	Fresh	3 hours	4 days	7 days	10 days	13 days	15 days	338 days
Severe mosaic:								
In vitro	+	+	+	+	+	+	+	−
In dry tissue	+	+	+	+	+	+	+	+
Enation mosaic:								
In vitro	+	0	0	0	0	0	0	0
In dry tissue	+	(2)	0	0	0	0	0	0

[1] Summary of results obtained in 3 series of tests.
[2] No tests were made with plant tissue.

Results reported in table 4 show that the enation virus was inactivated in less than 3 hours in vitro and in less than 4 days in dry plant tissue, while the severe mosaic virus remained active in vitro for at least 15 days and in dry plant tissue for 338 days.

TABLE 5.—*The effect of dilution upon infectivity of pea mosaic viruses* [1]

Virus tested	Infection obtained on peas at dilution of —								Check plant tissue inoculum [3]
	Check (no dilution) [2]	1:10	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	Check at end of inoculation [2]	
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Severe mosaic	100	100	100	100	25	5	0	100	100
Enation mosaic	2	0	0	0	0	0	0	0	24

[1] Juice extracted in hydraulic press from Perfection peas and inoculated to Perfection peas had a value of pH 6.3.
[2] Check plants in second column inoculated soon after juice was extracted. Check plants in ninth column inoculated following inoculation with diluted extract 3 hours after juice was extracted.
[3] Inoculated by rubbing macerated plant tissue on Perfection pea plants.

Results reported in table 5 show that the severe mosaic virus retains a low percentage of infectivity at 1 to 100,000 dilution, but is inactivated at 1 to 1,000,000 dilution. The enation mosaic virus is inactivated very rapidly in extracted plant juice, only 1 plant out of 50 becoming infected with the freshly extracted juice. The lack of infection with the diluted juice may, accordingly, be attributed to lack of longevity in vitro or to the effect of dilution. It is also shown in table 5 that a higher percentage of infection is obtained by fresh plant-tissue inoculation than with extracted plant juice containing the enation mosaic virus. A relatively low percentage of transfer of the enation mosaic virus (10 to 25 percent) as compared to that obtained with the severe mosaic virus (100 percent) by the macerated-plant-tissue-carborundum method, has been consistently observed throughout these investigations.

The severe mosaic virus was inactivated at temperatures between 60° and 70° C. (table 6). No infection was obtained by inoculations with the enation mosaic virus exposed to temperatures from 40° to 80°. The short longevity of this virus in extracted plant juice may account for this lack of infection.

TABLE 6.—*Inactivation of pea mosaic viruses following inoculation with virus extract* [1] *exposed to heat for 10 minutes*

Virus tested	Check	40° C.	50° C.	60 °C.	70° C.	80° C.	Check [2]
Severe mosaic	+	+	+	+	0	0	+
Enation mosaic	+	0	0	0	0	0	0

[1] Plant juice extracted in hydraulic press, pH 6.3, was treated in thin-walled glass tubes in temperature-controlled water bath for 10 minutes.

[2] Check plants inoculated after other inoculations had been made, which was 3 hours after juice was extracted.

SUMMARY

Two virus diseases, designated as enation mosaic and severe mosaic, often develop destructively in pea plantings in the State of Washington. The greatest damage from these viruses has been noted on market-garden and canning peas in the western part of the State.

Seed transmission tests have shown that the two viruses are rarely carried in the seed.

The severe mosaic has a wide host range among leguminous plants; the host range of the enation mosaic virus is much more limited.

The two viruses differ in the character of the symptoms produced on affected plants; in tolerance to dilution; in longevity in vitro and in dried plant tissue; in inactivation by exposure to heat; and in percentage of infection resulting from mechanical inoculation.

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U. S. GOVERNMENT PRINTING OFFICE: 1937